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UNIVERSITY OF ALBERTA

THE FUNCTIONAL ROLE OF VOLTAGE-SENSITIVE CALCIUM CHANNELS IN SKELETAL MUSCLE

BY

AHMET MURAT ÖZ

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

PHARMACOLOGY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1992

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ISBN 0-315-77245-X

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DEGREE: Doctor of Philosophy

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Çalışmalarım sırasında bana yardımlarını esirgemeyen,

karım Leylâya,

annem Düriye,

beni yetiştiren teyzem Süreyya

ve sevgili anneannem Nezaket'e olan sevgi ve saygılarımın

bir parçası olarak, bu çalışmayı onlara ithâf ediyorum.

Murat Öz

"Vandal yürek! Görünki alkışlanasın.
Ez bütün çiçekleri kendine canavar dedirt
Haksızlık et, haksız olduğun anlaşılsın
Yaşamak bir sanrı değilse, öç alınmak gerektir"

İsmet Özel
Esenlik Bildirisi

ABSTRACT

The effects of calcium-free Ringer's solution and organic calcium channel blockers on twitch and tetanic responses were investigated in frogs skeletal muscle fibres. In the presence of calcium-free solutions containing low concentrations of EDTA, the area of the tetanic tension X time plot was greatly depressed, but twitch responses either increased or did not change in a 10 min recording period. At high concentrations of EDTA, both twitch and tetanic responses were abolished.

Similarly, the organic calcium channel blockers nitrendipine and verapamil both greatly depressed the area of the tetanic tension X time plot. Nitrendipine, at all concentrations used, either potentiated or did not change the maximum amplitudes of twitch responses. On the other hand, at very high concentrations, verapamil, in addition to its effect on tetanic areas, also depressed twitches.

Intracellularly recorded repetitive action potentials were unchanged in the presence of nitrendipine at all concentrations. The amplitudes of late after potentials were either not changed or slightly increased with the application of nitrendipine. The effect of verapamil on repetitive action potentials was of a complex nature. At relatively low concentrations, which caused a significant depression of areas, verapamil did not affect the repetitively occurring action potentials. At high concentrations of verapamil, use-dependent blockade of sodium-action potentials was observed. The time courses and the use-dependent blockade of action potentials were also consistent with the mechanical recordings. An exception to this consistency was that at relatively low concentrations of verapamil, although the reversal of use-dependent blockade of action potentials by lower stimulation frequencies was observed, tetanic contractions were not maintained.

Although it has been well established that transverse tubules of skeletal muscle fibres

contain high concentrations of voltage sensitive calcium channels, their functional significance was not known. During our studies, we found that pharmacological manipulations (reducing extracellular calcium ions and using the organic calcium channel blockers, nitrendipine and verapamil) that are known to suppress the activity of these calcium channels, also greatly depressed the maintained tension development during tetanic contractions. Since in physiologically functioning intact muscle fibres, repetitively occurring action potential trains, rather than single action potentials, are generated for the development of maintained tetanic type contractions, we have suggested that the functional role of voltage sensitive slow calcium channels is to permit the entrance of extracellular calcium ions that are required for the maintained tension development during tetanic contractions.

Since it was our hypothesis that the late after potentials that occur during tetanic stimulations in T-tubules can cause the opening of voltage sensitive slow calcium channels during tetanic contractions, we further studied the role of voltage sensitive slow calcium channels in T-tubule membrane preparations. It was found that by depolarizing the T-tubule vesicles to the range of late after potentials that occur during tetanic responses, there was a large calcium flux response. These $^{45}Ca^{2+}$ flux responses were blocked by the inorganic calcium channel blockers, Co^{2+}, Ni^{2+} and La^{3+}. The organic calcium channel antagonists, nifedipine and verapamil also blocked $^{45}Ca^{2+}$ flux responses but nitrendipine had no effect on these responses.

The calcium channel agonist Bay K8644 increased the $^{45}Ca^{2+}$ flux responses occurring in the range of late after potentials in T-tubule vesicles. Stereospecific enantiomers of (±)-SDZ 202-791 had opposite effects on these flux responses. Although (-)-SDZ 202-791 blocked $^{45}Ca^{2+}$ fluxes, (+)-SDZ 202-791 had an agonist effect on flux responses, indicating the stereospecificity of the effects of different enantiomers on $^{45}Ca^{2+}$ fluxes in T-tubule vesicles.

These results gave further support to our previous suggestion that the voltage sensitive slow calcium channels can play important roles during the late phase of tetanic contractions.

ACKNOWLEDGEMENTS

To Dr. George B. Frank, my supervisor and mentor, I would like to express my utmost appreciation. This thesis would not have been possible without his invaluable guidance, encouragement, and support. He was vital to my development as a scientist.

I am also very grateful to Dr. Susan Dunn, my co-supervisor for her continued support, friendship and invaluable scientific feedback on my work.

I gratefully acknowledge the criticisms and encouragement of the other members of my research committee - Dr. Bill Dryden, Dr. Peter Smith, Dr. Tessa Gordon, Dr. Richard Stein and Dr. Julio Vergara.

I extend my heartfelt thanks to all my colleagues and friends for their suggestions and assistance, especially Tushar Kokate, Manjunatha Bhat, Frances Sharon and Rory McQuiston. Special thanks to Joan McGinnis for her patience and excellent typing efforts, and untiring technical assistance. I would also like to thank Jaclyn Peebles and Greg Morrison for the preparation of the Figures.

Finally, I would like to thank the Alberta Heritage Foundation for Medical Research for their financial support.

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ABBREVIATIONS

e-c	excitation-contraction
F	Faraday's constant
G	giga
Hz	Hertz
K_d	dissociation constant
LAP	late after potential
l	litre
m/s	meters per second
m	milli
mm	millimeter
ms	millisecond
MW	molecular weight
n	number
nM	nanomolar
Osm	osmole
p	pico
R	resistance (ohms)
r	radius or radii
rpm	rotation per minute
SR	sarcoplasmic retriculum
τ	time constant
TnC	troponin C
TnI	troponin I
TnT	troponin T

T-tubule	transverse tubule
s	seconds
n	nano
μ	micro
VSCC	voltage sensitive calcium channels
VSSCC	voltage sensitive slow calcium channels

DRUGS AND CHEMICALS

ACh	acetylcholine
Ag/AgCl	silver-silver chloride
ATP	adenosine triphosphate
$BaCl_2$	barium chloride
$CaCl_2$	calcium chloride
$CoCl_2$	cobalt chloride
cam kinase II	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DHP	dihydropyridine
DMSO	desmethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene-bis(oxyethylenenitrilo)tetraacetic acid
dTC	d-tubocurarine
GTP	guanosine triphosphate

GTP-γ-S	guanosine-5'-[3-thio]-triphosphate
HCl	hydrochloric acid
IP_3	inositol trisphosphate
KCl	potassium chloride
$LaCl_3$	lanthanum chloride
LAP	late after potential
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
$NiCl_2$	nickel chloride
PKC	protein kinase C
PKIs	protein kinase inhibitors
PTX	pertussis toxin
TEA	tetraethylammonium
Tris-HCl	Tris(hydroxymethyl)aminomethane-HCl
TTX	tetrodotoxin

1. INTRODUCTION

'Ja calcium, das ist alles'

Otto Loewi, 1959

Calcium (Ca)

Atomic weight 40.08

Atomic number 20

Melting point 842°C - 848°C

Boiling point 1487°C

Valency 2

1.1 General Comment

Calcium (Latin calx: Lime), following its discovery by Humphry Davy in 1808, took its place as one of the most abundant alkali earth metal ions among the group II elements in the periodic table. Not until the late 1800s was its importance first mentioned in biological systems. Thanks to the tap water of London, supplied by the New River Water Company, Sidney Ringer was the first person to show that calcium was required for contraction of the frog heart (1883). In later studies, it was observed that, in Ca^{2+}-free solutions, electrical activity still persisted (Locke and Rosenheim, 1907; Mines, 1913) and that the strength of contraction was proportional to the calcium concentration in the bathing medium (Bay et al., 1933).

The first direct evidence for the involvement of Ca^{2+} in skeletal muscle contraction came from Heilbrunn and Wiercinski (1947). They demonstrated that Ca^{2+} was the only physiologically occurring cation which would cause contraction when injected in low concentrations into bits of skeletal muscle fibres. These observations were subsequently confirmed by experiments in which Ca^{2+} was introduced into frog muscle fibres by electrophoresis (Niedergerke, 1955). The importance of Ca^{2+} as the coupling link between depolarization and contraction in skeletal muscle was first demonstrated by Frank (1958) and supported by later studies (Bianchi and Shanes, 1959; Curtis, 1966; Bianchi and Bolton, 1967). This link between depolarization and contraction has also been known as excitation-contraction (e-c) coupling. E-c coupling was originally proposed by Sandow (1952) to describe the series of events that, starting with the propagated action potential of its surface membrane, leads to the twitch contraction of a muscle fibre. These events are defined as a series of successive steps that are depicted schematically in Figure 1. It is well established (Huxley and Taylor, 1958) that an excitatory stimulus, i.e. an action potential, at the surface membrane of muscle fibres is conducted toward the interior along the transverse tubular system. It is also accepted (Bianchi

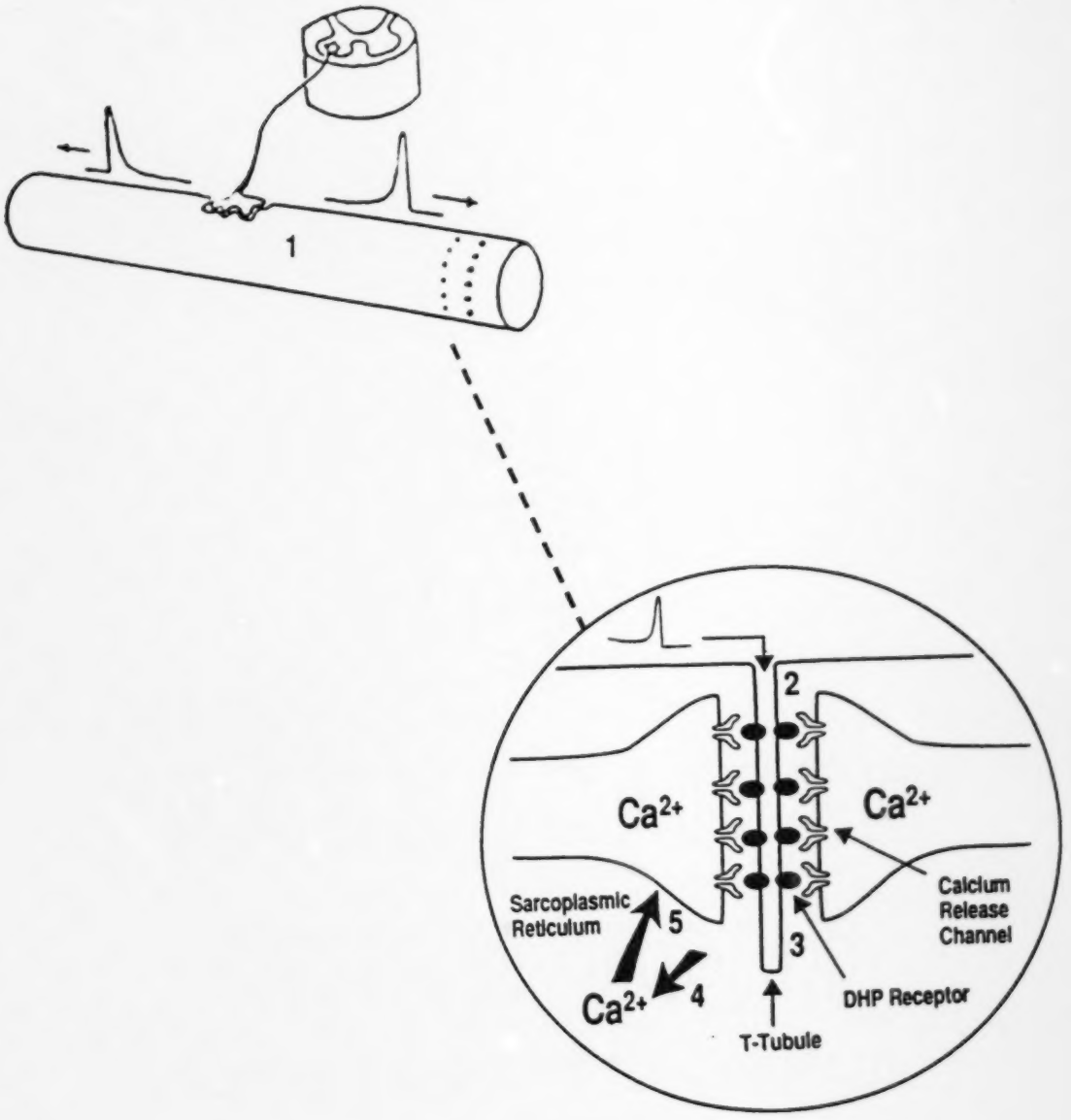


FIGURE 1: Schematic representation of the different steps involved in e-c coupling. Excitatory stimuli at the surface membrane (1) are conducted into the interior of the fibres along the transverse tubular system (2) and will cause the release of calcium from the sarcoplasmic reticulum (4). The released calcium will activate the contractile machinery and is subsequently taken up by the sarcoplasmic reticulum (5). Exactly how depolarization of the tubular membrane causes the release of calcium from the sarcoplasmic reticulum is not known (step 3).

and Shanes, 1959; Winegrad, 1970) that an action potential will cause a translocation of calcium from the sarcoplasmic reticulum to the myofilaments, resulting in muscle activation. In the past few years, major advances in understanding the molecular basis for e-c coupling have taken place with the identification, isolation and sequencing of two of the key proteins: a dihydropyridine (DHP) receptor which senses the T-tubule membrane potential and a ryanodine receptor which contains the calcium release channel in the sarcoplasmic reticulum, however, the mechanism of the coupling process between the T-tubule membrane potential and the release of calcium from the sarcoplasmic reticulum has yet to be elucidated (Figure 1, Step 3).

Regardless of the exact nature of this mechanism, the electrical events in transverse tubules are closely related to the mechanical responses of the muscle, and Ca^{2+} has a central place in this link.

1.2 Action Potential and its Role in Muscle Contraction

In vertebrate skeletal muscle fibres, the physiological event that leads to contractile activation is the action potential which consists of a spike followed by an early negative after-potential (Fig. 2). The upstroke of the action potential which has a maximum rate of rise to +400 to +700 V/sec is caused by a regenerative increase of Na^+ conductance while repolarization results from an inactivation of the Na^+ conductance and a delayed increase of the K^+ conductance. Thus the overshoot potential (see Fig. 2) and maximum rate of rise of the action potential depend linearly on the logarithm of $[Na^+]_o$, and the action potential is blocked by tetrodotoxin which specifically blocks the membrane Na^+ channels (Narahashi et al., 1964; Ferroni and Blanchi, 1965). Later, voltage-clamp studies of frog twitch fibres also demonstrated a transient inward Na^+ current following depolarizing steps; this inward current is also abolished by tetrodotoxin or by the removal of extracellular sodium and could be inactivated by a

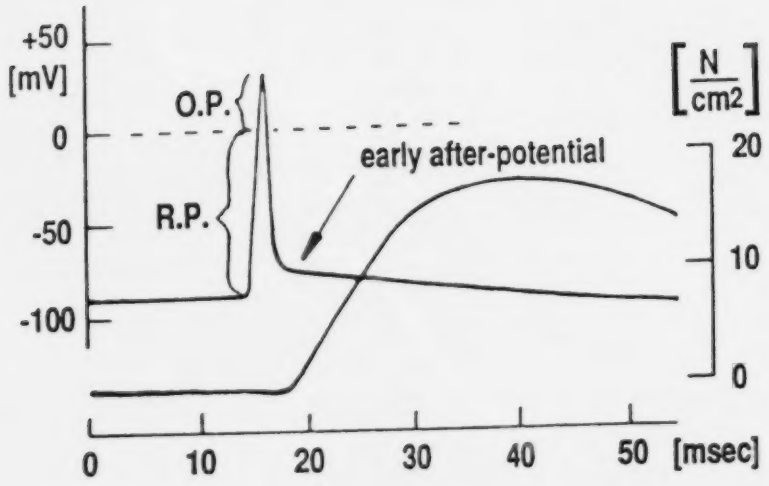


FIGURE 2: Action potential and isometric tension of a single skeletal muscle fibre of the frog. t = 18°C. O.P., Overshoot potential; R.P., resting potential (-90.3 mV in this fibre). A simultaneous record of action potential and tension from an isolated fibre was first published by Hodgkin and Horowicz (1957) and the figure was modified from their representation.

conditioning depolarization (Adrian et al., 1970a; Ildefonse and Roy, 1972). As in axons, the initial sodium current is followed by a delayed outward K^+ current, which is greatly reduced by extracellular tetraethylammonium ions (Stanfield, 1970). This delayed K^+ channel has lower ion selectivity (30:1, K^+:Na^+) as compared with that of the K^+ channel (100:1, K^+:Na^+) which determines the resting potential (Adrian et al., 1970a,b). The increase in the delayed K^+ current during depolarization brings the potential back to the equilibrium potential for these channels (-70 to -80 mV). The subsequent slow return to the resting potential brings about the early after-potential which is typical of skeletal muscle fibres (see Fig. 2). The early after-potential of frog skeletal fibres is about 20 mV in amplitude immediately after the spike component and gradually decays to the resting potential with a half-time of 10-20 msec (20°C) (Frank, 1957). This gradual decay depends on the time constant of the total fibre membrane (Frank, 1957).

In simultaneous recording of the action potential and contraction, it was clearly shown that the action potential preceded the beginning of the mechanical response by 1 or 2 ms (Hodgkin and Horowicz, 1957; Buchtal and Sten-Knudsen, 1959). This time interval is considered to represent the period during which e-c coupling occurs (Sandow, 1947). A.V. Hill (1948) proposed that diffusion of an activator substance released from the surface membrane during an action potential was too slow (2.5 sec for a small molecule freely diffusing across a fibre radius of 50 μM) to account for the relatively short time interval of 1-2 ms during e-c coupling.

By the mid 50's, although it was clear that an action potential occurred at the surface membrane and that it is followed, after a few milliseconds, by contraction of the myofibrils. The problem of the mechanism by which this change of surface membrane potential spreads into the muscle fibre remained unsolved. Definitive answers came from the combined use of different experimental techniques.

1.3 Inward Spread of Activation

The experiments of Huxley and Taylor (1955) suggested that excitation spreads along some structures within the Z line from the cell surface to the innermost myofibrils. These investigators produced highly localized depolarizations by applying current pulses, small enough so as not to trigger an action potential, through extracellular micropipettes located near the surface of isolated frog muscle fibres. They found that when the pipette tip was opposite the Z line, local contractions of the adjacent two half-sarcomeres occurred. The extent of contraction spread into the fibre depended on the strength of current. In addition, there were sensitive spots located around the perimeter of the fibre at the Z line level. In 1959 Huxley presented the electron micrographic evidence showing that the tubular structures at the level of the Z line were continuous from the surface to the centre of the muscle fibre. In the same year Andersson-Cedergren demonstrated similar findings and called this element the transverse (T) system. Finally, Peachey in 1965 clearly confirmed the continuity of the T-system (Fig. 3).

1.3.1 Ultrastructure of the T-System in Skeletal Muscle Fibres

The electron microscopic studies of Porter and Palade (1957) described a distinct structure composed of a central element and two lateral sacs. Because it was formed by three units it was named the triad. A few years later Andersson-Cedergren (1959) demonstrated that the triads were composed of morphologically distinct structures; transverse tubules (T-tubules) flanked by two lateral sacs that were part of the sarcoplasmic reticulum. T-tubules consist of tubular extensions of the surface membrane which enter at the level of the Z line in frog muscle fibres. The flattened tubules are 30-80 nm in diameter and form a continuous network (see Fig. 3). They penetrate into the fibre along a line vertical to the fibre axis into the spaces between myofibrils, thereby surrounding or totally enwrapping each fibril and finally ending in a blind

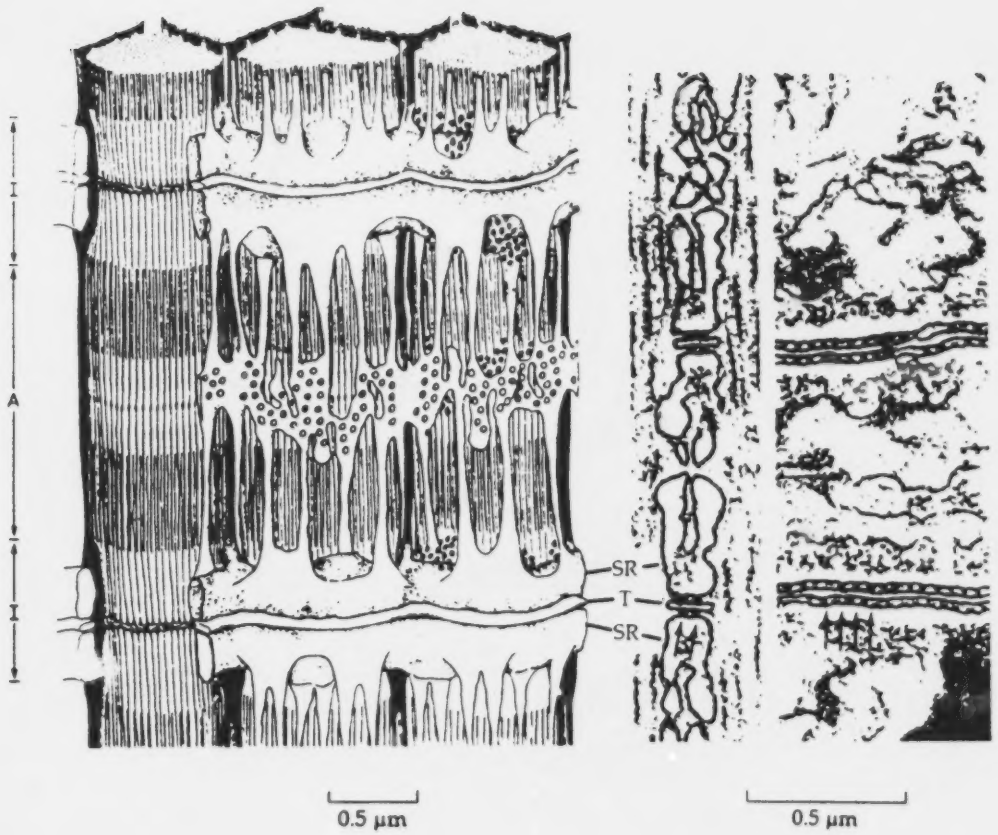


FIGURE 3: A three-dimensional reconstruction of the internal membrane system (transverse tubular system and sarcoplasmic reticulum) in association with the individual myofibrils of a muscle fibre and combined electron microscopic representation of triadic junction. On the left, parts of five intracellular myofibrils are shown. A, A band; I, I band. [Modified from Peachey, 1965]. On the right, electron micrographs of fish muscle showing SR and T-tubules cut in cross section and longitudinally. Electron-dense feet can be seen at regular intervals (arrows). [Modified from Franzini-Armstrong and Nunzi, 1983].

sac. The tubules of the T-system communicate with the extracellular medium as demonstrated by showing that relatively large extracellular "marker molecules" such as the protein ferritin (11 nm in diameter), could penetrate into the lumen of the T-tubules where they could be localized by electron microscope techniques (H.E. Huxley, 1964).

1.3.2 Electrophysiological Properties of the T-Tubule System

Although most of the ultrastructural characteristics of the T-tubule system were revealed in electron microscopy studies, the question of whether the T-tubule membrane, like the surface membrane, is selectively permeable to ions was uncertain. In an attempt to solve this problem, Hodgkin and Horowicz (1960a,b) reasoned that if a sudden change in external concentration of a particular ion affected the outside membrane, it would produce an almost immediate change in membrane potential. Since the concentration of an ion in the T-tubule space requires time to equilibrate with the external solution after a sudden change to a solution containing a different concentration of the same ion, there should be a slower change in membrane potential during the washout period if the tubule membrane is permeable to the ion. In other words, if the tortuous T-tubule pathways that cause a restricted diffusion of extracellular ions, would not have existed, with the similar surface area of muscle membrane, the potential change of muscle fibre would follow the membrane time constant ($\simeq$ 20 ms). This quick, expected repolarization, after changing high K^+ solutions to low K^+ solutions, is not observed in skeletal muscle fibres since the time required for the diffusion of high concentration of K^+ remaining in T-tubules after high K^+ solution-treatment, to the changed low K^+ solution outlasts the time constant of the membrane. In their studies on isolated frog muscle fibres, they clearly showed that when $[K^+]_o$ was increased at a constant $[K^+]_o \times [Cl^-]_o$ product, depolarization was reached in 1-2 seconds, and thereafter remained constant. However, during a fast washout period that took a fraction of a

second, repolarization followed a much slower time course than would be expected from the time constant of the muscle. Depolarization responses to a decrease and increase of $[Cl^-]_o$ had very similar time courses. These results supported the idea that the T-tubule membrane is permeable to K^+ ions and not permeable to Cl^- ions, whereas the surface membrane is permeable to both. Furthermore, the larger the $[K^+]_o$, the slower the response to a sudden return to low K^+ concentration. It was also found that if fibres of different diameters were exposed to the experimental solution, the larger the diameter, the slower the time course of the repolarization. When the volume occupied by K^+ ions was estimated from the repolarization responses, it corresponded to 0.2%-0.5% of the fibre volume. Later, Peachey (1965) calculated from electron micrographs that the T-tubule volume is approximately 0.3% of the fibre volume. Confirming these results, Nakajima et al., 1973, showed that this slow repolarization phase disappeared after the T-tubules were disconnected from the sarcolemma by a glycerol treatment. In many subsequent studies, Hodgkin and Horowicz's original approach to calculate the concentration of K^+ ions in T-tubules was extensively used to calculate the concentration of K^+ ions accumulated in T-tubules during repetitive activities of muscle fibres (Freygang et al., 1964a,b; Kirch et al., 1977). Accumulation of K^+ ions in the T-tubules during tetanic stimulations was first indicated by the work of Freygang et al. (1964a). They studied the long lasting depolarization called late after-potentials (LAPs) which occur after muscle fibres are stimulated tetanically. Freygang et al. (1964a,b) suggested that the LAP is caused by an accumulation in the T-tubules of K^+ ions which leave the fibre during each successive action potential. In subsequent studies, it was demonstrated that after the detubulation of muscle fibres by the glycerol treatment, the LAP was completely abolished (Gage and Eisenberg, 1969b). This finding confirmed the earlier studies suggesting the T-tubules as the site for the accumulation of K^+ ions during tetanic stimulations. Due to the diffusion delays caused by the long, tortuous and narrow tubular system, the K^+ ions

take some time to be reduced to the normal extracellular K^+ concentration. Thus the observed slow return of the LAP ($t_{½}$ = 350 msec) (Freygang et al., 1964a) to resting potential can be explained by the slow diffusion of K^+ out of the T-tubules to the exterior of the muscle fibre (Nakajima et al., 1969, 1973). This was also confirmed by the experiments of Kirsch et al., (1977). They showed that the slow decline of the LAP and K^+ repolarization (Nakajima et al., 1973) occurred at similar rates and confirmed that both phenomena could be explained by the accumulation of K^+ in T-tubules.

In addition to late after potentials, the membrane capacitance of muscle fibres has also been explained in terms of properties of the T-tubular system. In early studies on the linear electrical properties of frog skeletal muscle fibres it was found that the value of specific membrane capacitance (about 6 $\mu F/cm^2$) was considerably larger than expected for a layer of plasma membrane, which in nerves has a capacitance of only 1 $\mu F/cm^2$. It is now clear that this difference is largely due to an underestimate of the total membrane area in a unit length of muscle fibre. Electron microscope studies show that in a typical frog fibre the surface area of the transverse tubular membranes is roughly 5-7 times as large as the area of the sarcolemma, which is generally used for the calculation of specific capacitance (Peachey, 1965). This increased specific capacitance has important consequences on the electrical properties of muscle fibres. Since the time constant is the product of total membrane capacitance and the input resistance of muscle fibre, any increase in membrane capacitance will also cause an increase in the time constant of the membrane. As discussed above, the membrane time constant is the major determinant of the time course of the early after-potential, typical of the muscle action potential (Frank, 1957). Disconnecting the tubular system by glycerol-shock treatment to reduce the tubular component of total fibre capacitance greatly reduces the early after-potentials (Gage and Eisenberg, 1969a,b).

1.3.3 Inward Conductance of Electrical Activity in T-System

As was described previously, during their pioneering experiments, Huxley and Taylor (1958) came to the conclusion that the signal for muscle contraction is mediated by the inward spread of depolarization via the T-tubule system. This conclusion has been further strengthened by the use of the glycerol-shock technique (Howell and Jenden, 1967). In fibres treated this way, the action potential still occurs at the surface membrane but there is little or no mechanical activity indicating the essential role for e-c coupling of continuity between the sarcolemma and the T-tubule system (Gage and Eisenberg, 1967; Eisenberg and Eisenberg, 1968; Howell, 1969).

In conclusion, these early studies have attracted the attention of many workers in this field to the T-tubular system as the structure that is responsible for the inward conduction of the muscle excitation. For many years the question had remained unsettled as to whether the inward spread of this excitation along the T-tubules is an active, regenerative process or whether it occurs in a passive, electrotonic fashion. The first evidence on the presence of active propagation in the T-system came from studies showing the temperature dependence of the radial spread of activation in single muscle fibres (Gonzales-Serratos, 1966). Constantin (1970) confirmed this by showing that a surface membrane depolarization just above the contraction threshold could cause shortening of the entire cross-section of a muscle fibre. He also showed that the radial spread of contraction could be diminished by reducing the extracellular sodium concentration or by adding tetrodotoxin (TTX) to the bath medium. This can be explained by the fact that depolarization of the muscle fibre can produce an increase in sodium conductance in the transverse tubular membrane, resulting in an inward sodium current, which contributes to the spread of a depolarization or action potential along the T-system. Gonzales-Serratos (1971) performed subsequent studies which supported their preliminary accounts of a regenerative process along the transverse tubules. Gelatin setting and longitudinal compression of a muscle

fibre made microscopic observation of straightening of individual myofibrils possible. High-speed cinemicrography recorded the time course of shortening of the myofibrils, from which measurements of velocity of inward activation were made. This was estimated to be approximately 7 cm/sec at 20°C, with a Q_{10} of 2.13. The Q_{10} value is similar to that for the conduction velocity of a muscle action potential (Eccles et al., 1941), and thus Gonzales-Serratos (1971) deduced that such a Q_{10} would be compatible only with an active process. Nakajima and Gilai (1980a,b) stained the internal membrane system of the muscle fibre with the potential-sensitive dye, merocyanine, thus microscopically visualizing the location, size and time course of the potential change in the T-tubule. The rate of inward spread was found to be around 6.5 cm s^{-1} (25°C) in Xenopus muscle fibres, and doubled with a temperature increase of 10°C. Such a high temperature dependence would not be expected if the conductance mechanism were passive. Again, this suggests that the transverse tubular system is excitable, and necessary for full activation of the twitch response. Therefore, the experimental evidence presented thus far confirms the concept that the inward transmission of excitation into the T-system of skeletal muscle fibres occurs through an active process rather than by passive electrotonic spread. Various studies have attempted to calculate the shape of the action potentials on the surface membrane and in the T-tubules from voltage clamp data in terms of the parameters of the Hodgkin and Huxley model of squid axon excitability (Adrian et al., 1970a,b; Adrian and Peachey, 1973). The results obtained in these studies have suggested that there is a transmission delay within the T-system, so that depolarization of surface membrane occurs before depolarization of the most axially located T-tubule membranes. This propagation lag (about 1 ms) between the surface action potential and T-system membrane potential changes has also been confirmed in optical studies using potential sensitive dyes (Nakajima and Gilai, 1980a,b; Vergara and Delay, 1986). Calculated action potentials in T-systems also have a slightly broadened action

potential duration (Adrian et al., 1970a). This slight action potential broadening that might gain some importance during repetitive action potentials was also shown in the optical studies made to observe potential changes in the T-system (Heinz and Vergara, 1982; Vergara and Delay, 1986).

The action potential, after a latency of only a few milliseconds (1 or 2), is followed by a brief contraction of the frog's toe muscle fibre which lasts about 100-150 ms. Such a phasic contraction of response is known as a twitch. To evaluate the role of the action potential in e-c coupling it would be necessary to determine the quantitative relation between membrane potential and tension output under a variety of physiological conditions, but this cannot be directly established for the twitch in terms of the potentials produced by the action potential itself since these continuously and rapidly change. Hodgkin and Horowicz (1960a,b) approached this problem by studying depolarization-induced contractures set up in single fibres of skeletal muscle. These investigators changed the membrane potential by altering the $[K^+]_o$ at a constant $[K^+]_o$ X $[Cl^-]_o$ product. The relationship between the membrane potential measured by a microelectrode inserted into muscle fibre and the logarithm of the potassium concentration in solution was linear as predicted from the Nernst equation. Force development, on the other hand, was a more complex function of the potential. Their results indicated the following: a) the threshold for force activation is normally reached near -50 mV; b) the extent of force development is related to the membrane potential by a steep sigmoid curve (activation curve); and c) force development appears to be maximal, i.e. saturated at a membrane potential around -20 mV. Studies with voltage-clamped short muscle fibres of the frog also confirmed these results (Caputo and Fernandez de Bolanos, 1979). These results also clearly demonstrated that a stimulus causing an "all-or-none" type of action potential exceeding +30 mV will always activate the contractile machinery fully and it will, therefore, result in an "all-or-none" type twitch response.

In addition to the supra threshold effect of membrane depolarization, maintained subthreshold potential changes across the plasma membrane can also cause some functional changes such as an increased O_2 consumption and heat production ("Solandt effect" Solandt, 1936), an increased resistance to stretch and an increase in the mechanical response to slightly higher K^+ concentrations (Vos and Frank, 1972a,b). In these small maintained depolarizations produced by subthreshold high K^+ concentrations, slow but continued Ca^{2+} influxes occur and produce an increased $[Ca^{2+}]_i$ (Bianchi and Shanes, 1959; Weiss and Bianchi, 1965; Blatter and Blinks, 1991).

So far, although the inward transmission of a regenerative action potential into the T-system is well established, the fundamental question of how the tubular action potential is linked to release of internal Ca^{2+} has not yet been resolved.

1.4 Movements of Calcium Ions in the Activation of Contraction

1.4.1 The Functional Ultrastructure of the Sarcoplasmic Reticulum (SR)

The sarcoplasmic reticulum which makes up less than 10% of the total volume of the twitch muscle fibres in frog, is an internal membrane system that surrounds the myofibrils, consisting of flattened sacs that extend from Z-line to Z-line which is also the length of a sarcomere functional unit of the muscle fibre (Peachey, 1965). The configuration of the SR varies along the length of the sarcomere, appearing as terminal cisternae in the region of the I-band and as longitudinal tubules and a fenestrated collar in the A-band region (Figure 3).

The SR of skeletal muscle consists of two morphologically and functionally distinct regions: the terminal cisternae which form the triadic junction with the T-tubules, and the longitudinal elements of SR, which are slender tubules that connect medially with two terminal cisternae, forming a continuous SR compartment. Membrane preparations of SR obtained from

muscle homogenates have been subfractionated by density-gradient centrifugation into fractions originating from T-tubule, terminal cisternae (heavy microsome), and longitudinal tubule (light microsome) (Meissner, 1975; Lau et al., 1977). The isolated T-tubules are characterized by a high cholesterol content, the presence of Ca^{2+}-ATPase, Na-K ATPase, ß-adrenergic receptors, isoproterenol stimulated adenylate cyclase activity and dihydropyridine-class Ca^{2+} channel ligand binding sites (Caswell et al., 1979, Hidalgo et al., 1986; Jaimovich et al., 1986; Dunn, 1989). The longitudinal tubules are highly enriched in Ca^{2+} pump membrane, which constitutes 90% of total protein. This protein is responsible for translocating Ca^{2+} ($K_{Ca^{2+}}$: 0.1 - 1 μM) from the myoplasm to the lumen of the SR by hydrolysing ATP; two Ca^{2+} ions are translocated per hydrolyzed ATP (Martonosi, 1984). Fractions originating from terminal cisternae, besides the Ca^{2+}-ATPase activity, contain three different proteins; ryanodine receptors (or Ca^{2+} release channels),inositol trisphosphate (IP_3) receptors and calsequestrin (see Fleischer and Inui, 1989; Tsien and Tsien, 1990). Calsequestrin is a hydrophilic protein, which binds large amounts of Ca^{2+} (40 mol Ca^{2+}:1 mol) with low affinity (K_D = 0.8 mM). It is presumed that it plays a Ca^{2+} storing role by complexing with the Ca^{2+} in the terminal cisternae (Carafoli; 1987). Indeed, in electron-probe studies, it was clearly shown that the terminal cisternae of SR contain as much as 50 mM Ca^{2+} and function as the main Ca^{2+} store in the muscle fibre (Somylo, 1977). The release of the Ca^{2+} stored in the terminal cisternae has been shown in a number of studies.

Winegrad, in his pioneering studies of autoradiographic calcium location (1965, 1970), qualitatively showed the loss of stored, radioactively labelled calcium from the terminal cisternae upon stimulation. A.V. Somlyo et al., (1981) determined the quantitative release by electron-probe analysis, and showed that the calcium content of the terminal cisternae decreased from 50 mM in resting muscle to 30 mM in fibres frozen during tetanic stimulation.

In agreement with previous studies, binding sites for ryanodine and IP_3 in heavy SR

fractions (Inui, et al., 1987; Hymel, et al., 1988) have shown to correspond to Ca^{2+} release channels gated by Ca^{2+} itself and IP_3. The release of Ca^{2+} from terminal cisternae of the SR by either Ca^{2+} or IP_3 has been shown using both skinned muscle fibres (Fabiato, 1985; Lea et al., 1986) and heavy SR vesicles (Inui, 1987; Hymel et al., 1988).

In conclusion, it appears that the main function of the SR is to regulate the availability of intracellular Ca^{2+} to the myofilaments for muscle contraction and relaxation. Different parts of the SR seem to have specific functions in the process of Ca^{2+} homeostasis. In terms of morphological and molecular structure, the terminal cisternae seem to be specifically oriented for the storage and release of Ca^{2+} to sarcoplasm. On the other hand, the longitudinal tubular part of the SR is more likely to be involved with the sequestration of Ca^{2+} into the SR.

1.4.2 Calcium-Force Relation During Twitch and Tetanic Contractions

Quantitative studies of whether the elevated intracellular Ca^{2+} concentrations produced by the release from SR is actually capable of inducing a contraction of contractile machinery have been carried out using chemically or mechanically skinned muscle fibres (Natori, 1954; Julian, 1971; Stephenson and Williams, 1981). In experiments with frog twitch muscle fibres, it was shown that the Ca^{2+} threshold concentration was approximately 1 μM and maximal isometric force was achieved at 10 μM (Hellam and Podolsky, 1969).

The most direct evidence for an increase in myoplastic $[Ca^{2+}]$ during contractile activation was obtained with optical methods, based either on absorption changes resulting from the binding of Ca^{2+} to various indicators such as metallochromic dyes or the light emitted by a Ca^{2+} sensitive photoprotein such as aequorin (Ashley and Ridgway, 1970; Allen and Blinks, 1978; Miledi et al., 1982). As a result of these studies it was found that the calcium ion concentrations of about 0.1 μM in resting muscle fibres may increase, in twitch responses, to 8 μM when determined by the

arsenazo method or 10 μM when determined by the aequorin method (Blinks et al., 1978; Miledi et al., 1982) in twitch responses. All Ca^{2+} transients, however, have the following in common: the peak of the transient coincides with the steepest part of the tension rise, and the maximum force is developed during the declining phase of Ca^{2+} transient. An important conclusion that can be derived from the combination of experiments on skinned fibres and the measurements of calcium transients, is that the free Ca^{2+} concentration (10 μM) established during a twitch is so high that it fully activates the actinomyosin system. Following an action potential, the contractile machinery ought to be maximally activated by this high Ca^{2+} concentration, thus giving rise to an all-or-none twitch concentration. An important consequence of this response is that since the concentration of calcium ions is always supramaximal in an all-or-none twitch response, force cannot normally be graded in a twitch muscle fibre by a cellular control mechanism. In the intact muscle, force can be graded with more "centralized" control mechanisms by motor units, each unit comprising one motor neuron and the group of muscle fibres that it innervates (Liddel and Sherrington, 1925).

In intact muscle within the body, the gradual force development depends on either the recruitment of new motor units and/or the increase of the frequency of stimulation by motor neurons (Burke, 1981). In terms of a single muscle fibre, modulation of the force output of the fibre by changing the frequency with which a motor neuron fires is important (Kernell, 1965a,b). During repetitive stimulation of muscle fibres, due to mechanical summation, the force developed by each fibre is greater than its twitch force.

Using aequorin light signals, intracellular Ca^{2+} concentrations during repetitive stimulations have been measured in different studies (Blinks et al., 1978; Allen and Blinks, 1979). In studies with aequorin light signals during isometric tetanus of frog tibialis anterior, Allen and Blinks (1979) showed that, at intervals of 160 ms, twitches are unfused and tension

fluctuates between high and resting values, indicating a corresponding fluctuation of free calcium (Blinks et al., 1978). When the frequency of stimulation is increased the $[Ca^{2+}]_i$ is maintained at a high level since the Ca^{2+} released from the SR cannot be entirely pumped back in the short intervals between stimuli. As each stimulus released more Ca^{2+}, the concentration of Ca^{2+} in the cytosol rises and eventually reaches a much higher value than during a twitch, giving rise to fused tetanus.

1.4.3 Calcium Sensing Proteins During Muscle Contraction

In biochemical studies, binding of Ca^{2+} to various intracellular proteins has been known for many years (Ebashi and Endo, 1968; Endo, 1977; Kretsinger, 1980). The EF-hand domain, first described in the crystal structure of parvalbumin, has been identified as a calcium binding domain in approximately 20 separate types of intracellular calcium binding proteins, including troponin-C, calmodulin, parvalbumin and the myosin light chains (Claudia et al., 1991). Each motif contains two α-helices oriented at approximately 90° and linked by a central loop with precisely spaced negatively charged amino acids binding Ca^{2+} to protein. In general, two distinct roles for these intracellular calcium proteins have been ascribed. Some are sensors of the intracellular calcium levels and include calmodulin and troponin-C. These interact with other proteins in a calcium-dependent fashion and allow muscle contraction and enzyme regulation (Parmacek and Leiden, 1991). A second group, which includes parvalbumin and intestinal Ca^{2+}-binding protein, is probably responsible for the maintenance of a buffered intracellular calcium concentration (Gillis et al., 1982). In skeletal muscle fibres, troponin-C has been shown to be the most important calcium sensing protein involved with the contraction.

Soon after the discovery of troponin as the Ca^{2+} binding site of the thin acting filaments by Ebashi, it was found that troponin comprises three protein subunits, troponin C (TnC),

troponin I (TnI), and troponin T (TnT) (Ebashi, 1974). TnC is the calcium sensing protein and has four Ca^{2+} binding sites (Sites I - IV from the N-terminal of the polypeptide chain in sequence). Sites I and II have low affinity for Ca^{2+}, and sites III and IV have high affinity for Ca^{2+} (Herzberg and James, 1985). The high affinity binding sites are also able to bind Mg^{2+} competitively (Potter and Gergely; 1975). Evidence to date suggests that the low affinity, Ca^{2+}-specific sites are directly involved in the regulation of muscle contraction (Johnson et al., 1979), while the high affinity, Ca^{2+}-Mg^{2+} sites may stabilize the troponin complex structure (Zot and Potter, 1987). TnI, another subunit of troponin binds to actin and inhibits actomyosin-ATPase activity. This inhibitory action of TnI is relieved by TnC when the latter is occupied by Ca^{2+}, whereas, by itself, TnI is always inhibitory. TnT, on the other hand, connects the other two subunits to tropomyosin which is an α-helical, rod-shaped molecule composed of two polypeptide chains in a coiled-coil configuration (Ebashi et al., 1969).

According to recent models of muscle contraction, Ca^{2+} binding to low affinity, Ca^{2+} specific binding sites on TnC produces conformational changes that are propagated throughout other thin filament proteins including TnT, TnI, tropomyosin and actin. As a result of these, inhibition of actomyosin ATPase by TnI and tropomyosin is relieved when Ca^{2+} binds to TnC.

1.5 Excitation-Contraction Coupling in Skeletal Muscle

1.5.1 Functional Ultrastructure of the Triadic Junction

As earlier described, the information flow between T-tubule and SR occurs within the triad which may be regarded as an "intracellular synapse". A few years later, in electron micrographs Andersson-Cedergren (1959) demonstrated that the triads were composed of morphologically distinct structures; T-tubules bordered by two lateral sacs which were part of the SR and are called junctional SR. In frog skeletal muscle, there is approximately a 15 nm gap

between the T-tubular membrane and the junctional part of the SR (Franzini-Armstrong, 1970). Later, more detailed electron microscopy studies showed that the electron-dense particles or "foot"-like structures which form a regular tetragonal lattice of about 800 "feet" per μm^2 junctional membrane protrude from the junctional part of SR through the gap (Franzini-Armstrong and Peachey, 1981).

From a number of studies it is known that the Ca^{2+} release channels are concentrated in heavy SR vesicles which are thought to be derived from the junctional SR (see Martonosi, 1984). Ca^{2+} efflux from SR vesicles is activated by caffeine, low concentration of ryanodine, Ca^{2+} and adenine nucleotides, and is inhibited by Mg^{2+}, ruthenium red, dantrolene and high concentration of ryanodine (see Palade et al., 1989). The release channels from the heavy SR vesicles have been incorporated into planar lipid bilayers (Smith et al., 1986) and shown to make a channel formation with a unit conductance of 100 pS with its respective pharmacological properties. Ryanodine, a plant alkaloid, binds to the Ca^{2+} release channels in the terminal cisternae (Fleischer et al., 1985). The ryanodine receptor, which has a molecular weight of 350-450 kDa, has been purified (Lai et al., 1988) and identified as the Ca^{2+} release channel by different groups who have incorporated the purified receptor into lipid bilayers (Imagawa et al., 1987; Lai et al., 1988). Electron microscopy of the purified ryanodine receptor showed particles with the characteristic size and tetrameric shape of the feet structures in the terminal cisternae, indicating that the receptor for the Ca^{2+} release channel and the foot are the same molecular structures (Saito et al., 1988). Recently, the ryanodine receptor has been cloned and its primary structure determined from cDNA sequencing (Takeshima et al., 1989).

In high resolution electron microscopy studies of T-tubules, small particles organized as tetrameric arrays consistently facing the underlying feet are apparent (Block et al., 1988). Although, based on the binding ratio of DHP receptors to ryanodine receptors (1:2), these

tetrameric particles are proposed to be composed of four DHP receptors facing every one of two feet structures in an array, the conclusive ultrastructure of T-tubules is yet to be established (Ashley et al., 1991). So far, no direct link has been found from electron microscopic studies though, nor has direct binding between the two isolated receptors been detected (Brandt et al., 1990).

Recently, although exciting developments have been made on the further understanding of the molecular structure of the triadic junction, the functional role of these opposing tetrameric structures of DHP and ryanodine receptors has yet to be known (Rios and Pizzaro, 1991). According to some of the proposals made in various studies it can be argued whether these tetrameric structures of DHP and ryanodine receptors represent a protected release site for a chemical trigger such as Ca^{2+} (Miyamoto and Racker, 1982) or are a direct contact site for so-called voltage sensors (Rios and Pizzarro, 1991) or are linked to each other by means of a third protein (Brandt et al., 1990; Kim et al., 1990).

1.5.2 Release of Calcium by SR Depolarization

Mathias, Levis and Eisenberg (1980) proposed a model of e-c coupling in which the SR membrane is electrically coupled to the T-tubule membrane, so that the action potential is propagated across the triadic junction to the SR membrane. This model implied that a change in the SR membrane potential would open voltage-dependent Ca^{2+} channels in the SR membrane. Further evidence for this model came from studies showing that both "native" and reconstituted SR Ca^{2+} channels possess strong voltage dependence (Smith et al., 1988; Stein and Palade, 1988). When Ca^{2+} release channels of SR are incorporated into lipid bilayers they showed a macroscopic I-V relation consistent with a voltage-dependent gating (Smith et al., 1985). The macroscopic conductance of Ca^{2+} release channels is significantly increased at positive potentials

(sarcoplasmic side of the SR is positive with respect to luminal side). This is not due to an increase in channel conductance, but to an increase in the channel open time. Similar behaviour has also been seen with native Ca^{2+} release channels in frog muscle SR vesicles ('sarcoballs') (Stein and Palade, 1988). Based on these findings, assuming that the T-tubule and the terminal cisternae are two electrically coupled structures, this model suggests that an action potential propagated down the T-tubule makes the SR potential more positive (sarcoplasmic side) and turns on the Ca^{2+} release channels on the SR (Mathias et al., 1980).

There have been a number of objections to these models. Firstly, the effective membrane capacity of frog skeletal muscle fibres is approximately 8 $\mu F/cm^2$ (Fatt and Katz, 1951) and this is just a value that would be expected from the surface area of the t-system (Peachey, 1965). If the SR membrane had been electrically coupled with the T-tubular membrane, the value of the effective membrane capacity of skeletal muscle fibres would have to be considerably larger than 8 $\mu F/cm^2$.

In addition, assuming that the release channel protein and the T-tubule membrane are in contact (or with a gap much narrower than the Debye length), even in the most favorable conditions in which a very low dielectric constant is assumed for the release channel protein, a substantial fraction of the potential decreases within the protein opposed to the membrane (Jordan et al., 1989). Even if part of the T-tubule membrane potential declined within the foot protein, this potential change would occur far from the SR membrane, and the bilayer experiments on the SR channels only demonstrate a sensitivity to voltage changes across the SR itself (see Rios and Pizzaro, 1991).

In conclusion, a depolarization of the SR or the voltage sensitivity of the Ca^{2+} release channel is unlikely to play a role in the e-c coupling transmission mechanism (see also Ruegg, 1988).

1.5.3 Charge Movement in the T-Tubular Membrane

During the initial studies of Hodgkin and Horowicz (1960a,b), the relation between membrane potential and tension was determined (see section 1.3.3). Schneider and Chandler (1973) proposed that in skeletal muscle, contractile activity is controlled by voltage-dependent intramembranous charge movements. This charge movement was recorded as an outward current upon depolarization where all known ionic currents are blocked and linear capacitance current is substracted. It is thought that the charge movement is delimited since the same amount of charge moves back upon depolarization. Two separate components of charge movement have been identified in skeletal muscle fibres: fast component (β) and a delayed component (γ). The origins of these components are yet to be resolved (Caille et al., 1985; Rios and Pizzaro, 1991, Dulhunty, 1992).

Following their initial studies, Chandler et al., (1976) proposed a physical "plunger" model for e-c coupling. In this model, a charged molecule in the T-tubule membrane is mechanically linked to a plug in the SR channel by a rod like, long plunger. When the plunger moves under the changing electric field, it operates the plug. In pharmacological studies, it has been shown that various Ca^{2+} channel antagonists, nifedipine PN200-110 and D-600, can inhibit charge movements as well as contractions under certain conditions (Hui et al., 1983, 1984; Lamb, 1986; Lamb and Walsh; 1987). Following these studies, Rios and Brum (1987) found that the block of charge movements by the Ca^{2+} channel antagonist, nifedepine is also accompanied by simultaneous reduction of Ca^{2+}-release flux, and proposed that the block affects the movement of the "voltage sensors" involved in Ca^{2+} release. The block was large only at the depolarized holding potentials. This was explained with a state-dependent binding scheme in which these Ca^{2+} channel ligands bind only to the inactivated states (Bean, 1984). On the basis of these observations, since the intramembrane charge movement has most often been associated with ion

channel gating (Hille, 1992), Rios and Brum (1987) proposed that the high affinity receptors for Ca^{2+} channel ligands in T-tubules, which may function as Ca^{2+} channel, can be considered as a candidate for the origin of charge movements and therefore called voltage sensors on a hypothetical basis.

On the other hand, the charge movements of the proposed DHP receptors are not specific for skeletal muscle. Recently there have been a number of studies identifying the charge movement for cardiac Ca^{2+} channels (Cannell et al., 1987), and linking to the e-c coupling in cardiac muscle fibres in which the importance of extracellular Ca^{2+} (Rich et al., 1988) and the mechanism of Ca^{2+} induced Ca^{2+} release is well established (Fabiato, 1983). Secondly, so far, there is no sound evidence on the direct contact between T-tubules and terminal cisternae. In addition, Ca^{2+} channel antagonists at the concentrations that blocks the charge movements (Eisenberg et al., 1983; Lamb and Walsh, 1987) either do not reduce or potentiate twitches in amphibian (Frank, 1984) or mammalian skeletal muscle (Frank et al., 1988; Singh and Dryden, 1988). Therefore, it would seem that these charge movements are not required for e-c coupling during twitch responses in skeletal muscle under more physiological conditions.

1.5.4 Chemical Transmission Hypotheses

The geometry of the triad in the living skeletal muscle fibre seems particularly suited for a chemically mediated process. Both the existence of a narrow gap (15-16 nm), kept constant during contraction, as well as the concept of a restricted space enclosed by the T-tubule and the junctional face of the terminal cisternae are favourable morphological features for chemical transmission. The diffusion of transmitters between the T-tubule and the SR membrane is readily possible in the microsecond range (Vergara *et al.*, 1985; Jaimovich, 1991).

1.5.4.1 IP_3 Induced Calcium Release of SR

IP_3 was first suggested as a second messenger in e-c coupling in skeletal muscle by Vergara et al. (1985) and Volpe et al. (1985) on the basis of Ca^{2+} releasing effects of IP_3 in respectively, skinned and fractionated SR. In support of this hypothesis are the observations that 1) the enzymes required for IP_3 synthesis and degradation are present (Vergara et al., 1985, 1987; Hidalgo and Jaimovich, 1989), 2) that IP_3 production is increased during tetanus (Vergara, 1985), and 3) that IP_3 can activate ryanodine-sensitive SR Ca^{2+} release channels in lipid bilayers (Liu et al., 1989; see also for a recent review Jaimovich, 1991).

Although some groups were unable to confirm the ability of IP_3 to release Ca^{2+} from the SR of skinned fibres (Lea et al., 1986) and intact fibres (Blinks et al., 1987), others have obtained positive results (Donaldson, 1987; Rojas, 1987). However, even when obtained, the response to applied IP_3 was always very much slower (at least 1-3 orders) than could be produced by caffeine or by Ca^{2+} in skinned fibres. In addition, the concentrations of precursors or enzyme activities such as PLC appear to be too low for the required release of IP_3 (Hidalgo and Jaimovich, 1989). Moreover, SR Ca^{2+} release starts to decay 0.5 ms after membrane repolarization, whereas degradation of the IP_3 signal would be too slow by several orders of magnitude (Somlyo, 1988).

In conclusion, although the modulatory role of IP_3 on the release of Ca^{2+} from SR is quite possible, the exact role of IP_3 in skeletal muscle e-c coupling remains somewhat controversial.

1.5.4.2 The Trigger Calcium Hypothesis

Heilbrunn and Wiercinski (1947) were the first to show that Ca^{2+} was the only physiologically occurring cation that would cause shortening when injected into bits of skeletal muscle fibre. In earlier studies of heart muscle, the importance of extracellular Ca^{2+} in e-c

coupling was also clearly shown (Ringer, 1883; Mines, 1913). These previous studies led Sandow (1952) to suggest that a depolarization or action potential along the muscle fibre promoted the entrance of calcium ions into the fibre and these Ca ions would subsequently initiate muscle contraction. In agreement with this hypothesis was the later reported influx of calcium ($\simeq$ 0.9 pmol/cm^2) during muscle activity (Bianchi and Shanes, 1959; Curtis, 1966) and the findings by Frank (1958) demonstrating that the removal of calcium from the bathing medium of skeletal muscle abolished its mechanical responses without affecting its electrical properties. However, the amount of Ca^{2+} entering into muscle fibre per twitch was roughly one hundred times less than the amount required for full activation (Frank, 1961; Curtis, 1966). In subsequent studies, Frank (1982b) showed that the elimination of twitches in Ca^{2+}-free solutions took 10-20 min as opposed to 15-45 sec that would be expected, if the free diffusion of Ca^{2+} out of the T-tubules took place. In addition, the lack of a relation between the length of the T-tubular network and the time required to eliminate the twitch further supported the idea that extracellular Ca^{2+} are bound to binding sites located on the extracellular side of the T-tubules (Frank, 1982b). These findings led to the development of the "trigger" Ca^{2+} hypothesis for e-c coupling in skeletal muscle (Bianchi, 1969; Frank, 1980). According to this hypothesis there are superficial binding sites on the luminal or extracellular surface of the T-tubular membranes which are occupied by Ca^{2+}. The presence of such extracellular binding sites for Ca^{2+} has been shown in various studies (Langer, 1982; Frank, 1982a,b; Brum et al., 1988). In addition to the extracellular binding sites, in a number of studies, intracellular binding sites for Ca^{2+} in different muscle fibres have also been found (Atsumi and Sugi, 1976; Suzuki and Sugi, 1978; Bers et al., 1986). In the trigger Ca^{2+} hypothesis, these Ca^{2+} ions bound to the intracellular surface of the T-tubular membrane are referred to as the "trigger Ca^{2+}". Some of these "trigger Ca^{2+}" can be released into the triadic junction by depolarization, produced by action potential or by some drugs (e.g.

acetylcholine). The Ca^{2+} ions diffuse across the triadic junctional space to the terminal cisternae of the SR where they stimulate the release of much larger amounts of Ca^{2+} into the myoplasm and initiate the mechanical response. The experimental findings showing the presence of a Ca^{2+}-induced Ca^{2+}-release mechanism in skeletal muscle fibre have given further support to 'trigger' Ca^{2+} hypothesis (Endo et al., 1970; Ford and Podolsky, 1970; Fabiato, 1985).

From the various hypotheses proposed to explain the process of e-c coupling in skeletal muscle, the 'trigger' Ca^{2+} hypothesis seems most plausible (see Frank and Oz, 1992). On the basis of this hypothesis, several actions of multivalent cations on contractions or contractures could easily be explained (Frank, 1980; Frank, 1986).

1.6 Calcium Channels

The concentration of free calcium ions in the cytoplasm is critically important for the control of many essential cellular functions like excitability, muscle contraction, as well as neurotransmitter and hormone release (Rubin et al., 1985). A diverse array of Ca^{2+} transporting systems function to maintain the steep concentration gradient between extracellular Ca^{2+}, known to be in the millimolar range, and intracellular Ca^{2+}, which can vary between 0.1-10 μM, depending on the state of the cell. Several different types of mechanisms serve to maintain the low concentration of intracellular Ca^{2+} by transporting Ca^{2+} either out of the cell or into intracellular storage sites (Carafoli, 1987).

The major pathway for Ca^{2+} entrance in many cell types is via plasma membrane calcium channels. Although the plasma membrane is normally virtually impermeable to Ca^{2+}, the opening of these calcium channels, due to the existence of a large Ca^{2+} concentration gradient, allows Ca^{2+} to move passively across the cell membrane down their electrochemical gradient into the cell.

Different types of calcium channels are known to exist and are characterized by fundamental differences in the mechanisms governing their opening and closing ("gating"). Some of these Ca^{2+} channels are gated primarily by changes in membrane potential. However, they may also be regulated by receptors, either through the action of diffusible second messengers and their phosphorylation systems or directly by the coupling of the channel to the receptor by means of a G-protein (Miller and Fox, 1990). These voltage sensitive calcium channels (VSCC) are discussed in detail in the following section, (see also Dunn et al., 1992).

Another class of Ca^{2+} channels are operated through receptor-dependent mechanisms. These channels are often referred to as receptor-operated Ca^{2+} channels and open in direct response to binding of an external ligand to an associated receptor (Bolton, 1979; Bentham and Tsien, 1987). Ca^{2+} channels that are opened by activation of ATP receptors and parathyroid hormone activated Ca^{2+} channels in osteosarcoma cells are some examples of receptor-operated channels (Bentham and Tsien, 1987; Yamaguchi et al., 1987).

Other types of Ca^{2+} channels include background channels and stretch-activated channels (Franco and Lansman, 1990; Fong et al., 1990). The Ca^{2+} influx through background channels is a result of the large electro-chemical gradient favoring the entrance of Ca^{2+} into the cell. The activity of such channels appears to be elevated in muscle fibres of mdx mice (mutant mice with muscular dysgenesis) and of individuals afflicted with Duchenne's muscular dystrophy (Franco and Lansman, 1990; Fong et al., 1990) and contributes to the elevation of $[Ca^{2+}]_i$ (Turner et al., 1988; Lansman and Franco, 1991). Although stretch-activated channels have been described in different types of cells, including endothelial cells, smooth muscle and skeletal muscle (Lansman et al., 1987; Kirber et al., 1988; Franco and Lansman, 1990), little is known about their functional characteristics.

1.6.1 Voltage-Sensitive Calcium Channels

Although VSCCs have traditionally been associated with excitable cells such as various types of muscle, neurons and endocrine cells, these channels have also been shown to exist in a number of non-excitable cell types, including various types of glial cells, myeloma cells and fibroblasts (Barres et al., 1988; Fukushima and Hagiwara, 1988; Villereal and Jamieson, 1988). In biophysical studies, the analyses of both whole cell and single channel current kinetics have provided deep insights into the gating properties of VSCCs. At least three different types of VSCCs have been identified in chick dorsal root ganglion (DRG) neurons and a fourth, P-type, has been described in cerebellar Purkinje cells (Nowycky, 1985; Tsien et al., 1988; Llinas, 1989). The main features of these different types of VSCCs are summarized in Table I.

The L-type VSCC, is the best characterized in terms of its gating properties. This channel has greater permeability to Ba^{2+} than to Ca^{2+} and it has a large unitary Ba^{2+} conductance (25 pS in 110 mM Ba). These channels have a high-voltage threshold for activation (positive to -30 mV) and conduct maximum current (peak current) in the voltage range of 0 to +10 mV (Swandulla and Armstrong, 1988). Although L-type channels in different tissues share similar current-voltage related features such as activation threshold and the voltage range of the peak amplitude (Beaty et al., 1987), their activation kinetics show significant tissue variability (Pelzer et al., 1990).

In addition to their high threshold of activation, L-type VSCCs are also distinguished by their slow and unique type of inactivation which is both voltage and Ca^{2+} dependent (Gutnick et al., 1989). Depending on membrane potential, temperature, the charge carrying ion and the tissue type, the inactivation time constant for neuronal L-type VSCCs varies between 30 ms to 1000 ms. The voltage-dependent nature of this inactivation is evident in the decay from its peak during a sustained depolarizing step. This voltage-dependence of inactivation of L-type channels

TABLE I

Electrical and Pharmacological Properties of the Four Types of Voltage Sensitive Calcium Channels*

	Low Voltage Activated Ca^{2+} Channel	High Voltage Activated Ca^{2+} Channels			
Channel Type	T	P	N	L (Neuronal)	L (Skeletal Muscle)
Single Channel Conductance (110 Ba)	~ 8 pS	~ 10-12 pS	~ 13 pS	~ 25 pS	~ 20-25 pS
Relative Conductance	$Ba^{2+} = Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$
Inorganic Ion Block	$Ni^{2+} > Cd^{2+}$	Cd^{2+} Sensitive	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$
ω-CgTx Block	Weak, Reversible	Resistant	Sensitive	Resistant	Resistant
Dihydropyridine Sensitivity	Resistant?	Resistant	Resistant	Sensitive	Sensitive
FTX Sensitivity	Resistant	Sensitive	Resistant	Resistant	-
Activation Range (for 10 Ca)[a]	Positive to -70 mV	Positive to -50 mV	Positive to -20 mV	Positive to -10 mV	Positive to -30 mV
Inactivation Range (for 10 Ca)	-100 to -60 mV	--	-120 to -30 mV	-60 to -10 mV	-50 to -10 mV
Inactivation Rate (0 mV, 10 Ca, or 10 Ba)	Rapid (tau ~ 20-50 ms)	Very Slow (tau ≈ 1 s)	Moderate (tau ~ 50-80 ms	Very Slow (tau > 500 ms)	Very Slow (tau ~ 2-10 sec) or no inact. at all[b]

[a] In 80 mM Ba^{2+} for P-type channels (Llinas et al., 1989).
[b] In lipid bilayers (Affolter and Coronado, 1985).
* From: Tsien et al., 1988; Pelzer et al., 1990, and Tsien et al., 1991.

is less pronounced than that of T currents (see below). However, unlike T channels, L channels can also be inactivated by a rise in intracellular free Ca^{2+} (Gutnick et al., 1989).

T-type calcium channels have also been identified and characterized in neurons and muscle cells. However, study of these channels is made difficult since their pharmacological properties have not been well differentiated. In general, they are resistant to organic Ca^{2+} channel antagonists (Porzig, 1990), although they are blocked by various inorganic blockers, being particularly sensitive to Ni^{2+} which blocks at low concentrations (40 μM) (Hagiwara et al., 1988). T current is characterized by a small single channel conductance of only about 5-10 pS (in 100 mM Ba^{2+}) and indeed this channel was originally named because of its "tiny" conductance (Nowycky et al., 1985). These channels are equally permeable to Ca^{2+} and Ba^{2+}. They can be activated at a low threshold range of -50 to -70 mV, and usually reach their peak amplitude in the range of -20 to -10 mV. Their activation kinetics are faster than L-type channels, leading to their description as "fast" Ca^{2+} channels by some authors (Bean, 1989; Hess, 1990). Unlike L-type current, T-current shows purely voltage and time dependent inactivation kinetics.

N-type Ca^{2+} channels, first described in chick DRG cells by Nowycky and colleagues (1985), are largely restricted to neurons, but their relative prominence varies significantly from one neuron to another. Although N-type channels are the dominant Ca^{2+} entry pathway in sensory, sympathetic and myenteric plexus neurons (Bean, 1989), they are mostly absent in cerebellar Purkinje cells (Tsien et al., 1991). This channel has a conductance intermediate between T- and L-type channels (13 to 17 pS in 110 mM Ba^{2+}). N-type channels, like L-channels, become activated at high thresholds. From a holding potential of -100 mV, strong depolarizations to -20 mV or more positive, elicit rapidly activating N currents in chick DRG. The N-type current differs from the L-type mainly by its greater sensitivity to holding potentials, and faster and strictly voltage-dependent inactivation kinetics (Nowycky et al., 1985).

Recently, P-type Ca^{2+} channels that appear to be particularly dominant in cerebellar purkinje cells, were described by Llinas and colleagues (1989). P-type channels are a novel class of high voltage activated Ca^{2+} channel that are activated over a range of potentials less negative than -50 mV and have a similar single channel conductance (14pS) to that of N-type channels. Their inactivation seems to be a slow process with a time constant of approximately 1 second. P-type Ca^{2+} channels are unlike L-type or N-type Ca channels in that they are insensitive to DHPs and ω-conotoxin GVIA. However, in cerebellar Purkinje cells and squid giant synapses, these channels can be potently blocked by components in the venom of the funnel web spider, *Agelenopsis aperta* (Llinas et al., 1989).

1.6.2 Pharmacology of L-Type Voltage Sensitive Ca^{2+} Channels

A large number of compounds have been shown to modulate the activity of L-type VSCCs. Several of these have therapeutic importance and are used to treat a variety of cardiovascular diseases (Triggle and Janis, 1987). These compounds have also been vital in guiding the identification and purification of VSCCs, as well as helping to understand more about their molecular properties (Hosey and Lazdunski, 1988).

Drugs that specifically interact with Ca^{2+} channels have been referred to as "Ca^{2+} channel ligands" (Triggle and Janis, 1987). Among these Ca^{2+} channel ligands, the ones that specifically bind and inhibit Ca^{2+} channels are collectively referred to as "Ca^{2+} channel antagonists" or "Ca^{2+}" channel blockers", and those capable of activating Ca^{2+} channels such as Bay K8644 and CGP 28392 are referred to "Ca^{2+} channel agonists" or "Ca^{2+} channel activators". In general these drugs have been the subject of extensive reviews (Godfraind et al., 1986; Triggle and Jannis, 1987; Porzig, 1990).

Ca^{2+} channel antagonists can be classified into at least three chemically distinct major

groups: a) the 1,4-dihydropyridine (DHP) derivatives such as nitrendipine and nifedipine; b) the phenyalkylamines such as verapamil and D-600, and c) the benzothiazepines, typified by diltiazem. These drugs act by binding with high affinity to distinct but allosterically interacting receptor sites located on VSCCs.

1.6.2.1 Mechanism of Action of Ca^{2+} Channel Ligands

The discovery of high-affinity binding sites for DHPs in membrane homogenates from excitable tissues, initially met considerable scepticism regarding their functional significance (Miller and Freedman, 1984), due to a large discrepancy existing between the apparent (nanomolar) affinity of the binding sites and the (micromolar) drug concentrations required for a pharmacological effect on Ca^{2+} channel function (Jannis et al., 1984). Electrophysiological evidence suggested that at least part of this discrepancy might be explained by the voltage-dependent conformational changes of the Ca^{2+} channel protein. The ability to block L-type channels is strongly affected by the holding potential in the voltage-clamp experiments. In these experiments, it was shown that the apparent binding affinity of resting and inactivated channels for the DHP derivative nitrendipine differed by a factor of more than 1000 (Bean, 1984). Therefore, binding constants measured in membrane fragments, where the potential has collapsed and all channels, presumably inactivated, cannot be compared with the apparent affinity of the drug under in vivo conditions. On the other hand, there was a very good correlation between binding affinities of Ca^{2+} channel blockers in brain, smooth muscle or heart homogenate and their relaxing effect on smooth muscle contractions (Janis et al., 1984; Janis, 1987; Godfraind et al., 1986), under conditions that binding and effect are both measured in depolarized preparations. To explain this dramatic effect of depolarization on DHP binding property, Bean (1984) postulated that DHPs bind preferentially to the inactivated state of Ca^{2+} channel than to the

resting state.

However, compared with DHPs, the channel blocking effect of phenylalkylamines and benzothiazepines is much more dependent on the stimulation frequency (i.e., "use-dependent") and much less dependent on the steady-state membrane potential (Lee and Tsien, 1983). Use-dependent block is induced by single or repetitive depolarization. The modulated receptor theory that describes the blockade of Na^+ channels by local anesthetics (Hille, 1977; Hondeghem and Katzung, 1977) has also proven to be a useful framework within which use-dependent Ca^{2+} channel block can be explained. According to this model, the binding and unbinding of organic blockers to their receptor sites in the channels are determined by channel state. Intracellular charged molecules can reach their target primarily via a hydrophillic pathway that is available when the channel is in the open state; uncharged molecules can access the channel via a hydrophobic pathway and therefore may bind to any state of the channel. Hence the use-dependent effect of verapamil and diltiazem seem to be related to their high degree of ionization at physiological pH values (the pK values of verapamil and diltiazem are 8.7 and 7.7, respectively). The ionized compound seems to need channel opening in order to reach their target site within the channel and to cause use-dependent blockade. An end result common to all three types of organic Ca^{2+} channel blockers is that drug-bound channels get locked in an unavailable state; repetitive depolarization increases the fraction of drug-bound unavailable channels, whereas rest periods at negative potentials facilitate the clearing of drug from the channels (McDonald et al., 1984; Uehara and Hume, 1985).

As mentioned previously, some of DHP derivatives including Bay K8644 and CGP 28392 have clear-cut capacity to enhance L-type Ca^{2+} channel current. Franckowiak et al. (1985) investigated the optical isomers of Bay K8644 and found that (-)-Bay K8644 potentiated the K_o^+-induced contractions of aortic rings, increased the contractility of perfused guinea pig hearts, and

lengthened the action potential of papillary muscle. By contrast, (+)-Bay K8644 had depressant effects on each of these parameters, suggesting that the dual action of the commonly used racemic preparation was due to the combination of an activator enantiomer and an inhibitory one. However, further examination of the enantiomers by Kass (1987) indicated that a single enantiomer could exert a dual effect, with holding potential being a key factor: (+)-Bay K8644 had a minor stimulatory effect on $I_{Ca,L}$ when rat or guinea pig ventricular myocytes were pulsed from -80 mV, and a marked inhibitory effect when pulsing was from -30 mV; (-)-Bay K8644 was a potent stimulator from -80 mV and a moderate blocker from -30 mV, suggesting that its activity was mainly responsible for the activity of the racemic compound. Such holding potential-dependent effects have also been described for DHP and phenylalkylanine group Ca^{2+} channel antagonists (Hess et al., 1984; Brown et al., 1986; McDonald et al., 1989).

1.6.3 Voltage Sensitive Ca^{2+} Channels in Skeletal Muscle

During early electrophysiological studies in frog skeletal muscle fibres, it was shown that slow Ca^{2+} action potentials can be elicited by using Cl^--free (methanosulfonate or acetate substituted), Na^+-free (sucrose substituted), high-K^+ solutions. Results in different studies showed that the rate of rise, overshoot and duration of these slow action potentials are a linear function of log $[Ca^{2+}]_o$ and are blocked by calcium channel blockers such as verapamil, D-600 and nifedipine (Beaty and Stefani, 1976; Nicola Siri et al., 1980; Kerr and Sperelakis, 1982, 1983). These early studies suggested that slow action potentials were produced by Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in skeletal muscle fibres (see also Sperelakis and Fabiato, 1985). When the Ca^{2+} inward current in frog muscle was studied by the voltage clamp technique (Stanfield, 1977; Sanchez and Stefani, 1978; Almers et al., 1981), elevation of $[Ca^{2+}]_o$ increased the inward slow current, which was depressed by Ca^{2+} channel blockers such as D-600,

nifedipine, and also by Ca^{2+}-free solutions.

In frog muscle fibres, during voltage clamp studies, Sanchez and Stefani (1983) showed that although the inward Ca^{2+} current has a dependence (with an activation threshold of -40 to -30 mv) similar to that of cardiac and smooth muscle voltage, its activation time course was more than an order of magnitude slower. For a depolarization from the holding potential of -90 mV to near 0 mV, it has a peak time of 100-200 msec after the start of the pulse, with a maximum value of 50-100 $\mu A/cm^2$ in the presence of 10 mM $[Ca^{2+}]_o$. It spontaneously decays during a maintained depolarization, with a time constant of about 1 second (20-23°C).

Different experiments indicate that Ca^{2+} channels are exclusively located in the membrane of the tubular system. Both slow Ca^{2+} action potentials and inward Ca^{2+} current can be abolished by glycerol-shock treatment, which dissociates the T-tubules from the surface membrane (Nicola Siri et al., 1980; Kerr and Sperelakis, 1982). Furthermore, in biochemical studies, using a radiolabeled form of dihydropyridines, it was found that the density of dihydropyridine binding proteins in T-tubule membrane preparations are 50-100 times higher than in any other tissue (Fosset et al., 1983; Glossmann et al., 1983).

The inactivation of this Ca^{2+} inward current during depolarization pulses is also much slower than the inactivation of this current in most other cell types (see Pelzer et al., 1990). Almers et al. (1981) concluded that the seconds-long process in frog fibres was due to depletion of Ca^{2+} in the T-tubules rather than due to voltage-dependent or Ca^{2+}-dependent mechanisms. It was also found that embryonic rat skeletal muscle myotubes with sparse T-tubules possessed slow Ca^{2+} currents that were hardly inactivated at all during depolarization pulses (Beam and Knudson, 1988). However, slow inactivation was apparent in adult rat muscle preparations with well-developed T-tubular systems. In line with these findings, single mammalian skeletal muscle Ca^{2+} channels in lipid bilayers do not inactivate at all (Affolter and Coronado, 1985; Coronado

and Affolter, 1985). On the other hand there is also evidence that a voltage dependent inactivation can be present in skeletal muscle (Sanchez and Stefani, 1983; Beaty et al., 1987). It was found that the inward Ca^{2+} current in intact frog fibres were inactivated in a voltage dependent manner. For example, in two-pulse experiments, inward Ca^{2+} current during the second pulse can be reduced without any detectable Ca^{2+} entry during the conditioning prepulse (Sanchez and Stefani, 1983; Cota et al., 1984). Similarly Beam and Knudson (1988) reported that long (30s) conditioning pulses to subthreshold potentials produced marked inactivation. Furthermore, the rate constant of decay of inward Ca^{2+} current monotonically increased with depolarization although the corresponding time integral of inward Ca^{2+} current followed a bell-shaped function (Cota and Stefani, 1989). The exact mechanism for the inactivation process in inward Ca^{2+} current has yet to be determined (Pelzer et al., 1990). It is possible that, as in cardiac inward Ca^{2+} current (Lee et al., 1985), more than one mechanism is employed simultaneously to produce inactivation of the inward Ca^{2+} current in skeletal muscle fibres (see Francini and Stefani, 1989).

In addition to this slow Ca^{2+} inward current, another component of Ca^{2+} current has recently been recognized in muscle fibres (Cota and Stefani, 1986; Garcia and Stefani, 1987). Like T-type current in cardiac muscle, it activates with a low threshold (about -60 mV) and very rapidly (within a few ms), however, it inactivates very slowly (over several seconds). This fast component of Ca^{2+} current is not sensitive to ...ledipine (Arreola et al., 1987).

1.6.4 Structure of Voltage-Sensitive Ca^{2+} Channel in Skeletal Muscle

The straight forward approach to the isolation of VSCC was to purify the channel-associated drug receptors for calcium antagonists by monitoring the binding activity of tritiated 1,4 DHPs. Since the T-tubule membranes from skeletal muscle have DHP receptors at a density

50-200 times higher than in any other tissue (Fosset et al., 1983; Glossman et al., 1983), they have extensively been used as the source for solubilization and purification of the DHP receptor. Initial studies determined that the DHP binding sites could be solubilized from skeletal muscle membranes in stable form with digitonin (Curtis and Catterall, 1983) or CHAPS (Borsotto et al., 1984). The solubilized DHP receptor, using lectin affinity chromatography on WGA-Sepharose and anion exchange chromatography, was first purified by Curtis and Catterall (1984). Purified DHP receptor preparations have been reconstituted in phospholipid vesicles or lipid bilayers by several groups and are shown to contain Ca^{2+} channel activity and their predicted pharmacological properties (Flockerzi et al., 1986; Curtis and Catterall, 1986; Smith et al., 1987). In most reports, a multisubunit complex of five distinct subunits [α_1(170 kDa), α_2 (140 kDa) which is disulfide linked to δ (30 kDa), β (55 kDa), and γ (32 kDa)] has been observed. Photoaffinity labelling studies have demonstrated that the α_1 subunit carries binding sites for DHPs and for other classical organic calcium channel blockers (Striessnig et al., 1986; Naito et al., 1989) and, in expression studies, this subunit has been shown to form a functional voltage-dependent calcium channel. It exhibits voltage-dependent Ca^{2+} current when a cell line lacking the other subunits was transfected with complementary DNA coding for the α_1 subunit (Perez-Reyes et al., 1989. The disulfide-linked α_2 and δ subunits are encoded by the same gene (DeJongh et al., 1990; Jay et al., 1991) and separate as a result of proteolytic event during processing. These subunits do not have appreciable hydrophobicity and may not, therefore, be integral transmembrane proteins. These subunits are, however, highly glycosylated and this underlies the usefulness of lectin affinity chromatography in the purification of the channel complex. The β subunit is neither hydrophobic nor glycosylated but, like the α_1 subunit, contains a site for phosphorylation by cAMP-dependent protein kinase. The γ subunit is glycosylated and has been proposed to be transmembrane (Catterall, 1988). A model, based on

those previously described (Catterall, 1988; Campbell et al., 1988; Dascal, 1990) is shown in Figure 4. The exact subunit structure and arrangement in the membrane, however, remains to be established. Recently, for example, it was proposed that the α_2 subunit is a peripheral protein that is exposed on the extracellular surface and is anchored in the membrane by the δ subunit (Jay et al., 1991).

All five subunits of the DHP binding protein from skeletal muscle have now been cloned and their cDNAs have been sequenced (Tanabe et al., 1987; Ellis et al., 1988; Ruth et al., 1989; Jay et al., 1990). The α_1 subunit displays 30-40% homology to the α_1 subunit of the voltage-dependent sodium channel (Tanabe et al., 1987). Like the sodium channel (Noda et al., 1984), this subunit has four internal repeats that exhibit sequence homology and each repeat contains six conserved hydrophobic stretches that are predicted to be transmembrane. The fourth transmembrane helix (S4) of each of these motifs contains positively charged amino acids (arginine or lysine) every 3rd or 4th residue and this region has been proposed to constitute the voltage sensing region of the channel protein (Catterall, 1988).

1.6.5 The Site of Action for Ca^{2+} Channel Ligands

In studies directed towards the identification of ligand binding sites on the α_1 subunit, a region in the putative cytosolic domain adjacent to the sixth transmembrane helix of the fourth homologous repeat (IVS6) was recently shown to be photoaffinity labelled by the DHP analogues, [^{3}H]-azidopine and [^{3}H]-nitrendipine (Regulla et al., 1991). These results suggest that the DHP binding site is intracellular, a suggestion that is both supported by the hydrophobicity of the DHPs and consistent with models in which it has been proposed that DHPs first partition into the lipid bilayer, prior to diffusing to their protein binding sites (Rhodes et al., 1985). However, this possibility is contradicted by electrophysiological evidence for an extracellular exposure of the

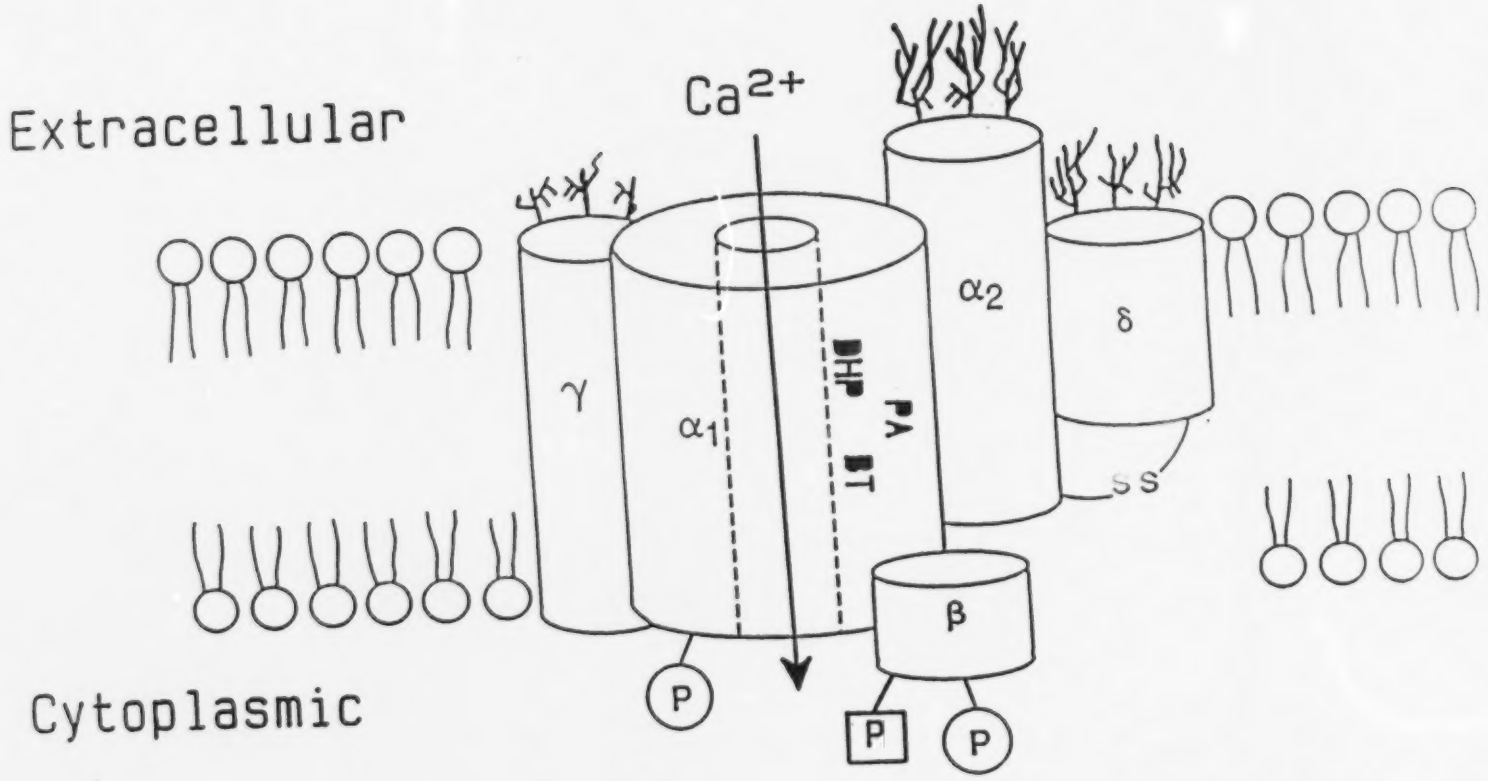


FIGURE 4: Proposed model for skeletal muscle dihydropyridine-sensitive calcium channel complex [modified from Dunn et al., (1992)].

DHP, dihydropyridine receptor; PA, phenylalkylamine receptor; BT, benzothiazepine receptor; S-S, disulphide bond; P, phosphorylation sites for protein kinase C (square) and cAMP-dependent protein kinase (circle).

DHP binding site. In experiments using the ionized, and presumably membrane impermeant, DHP derivatives, amlodipine and SDZ-207-180, the drugs were reported to be ineffective when applied intracellularly (Kass et al., 1991). In support of this observation, two recent studies employing photolabelling techniques (Nakayama et al., 1991; Striessnig, 1991) and subsequent sequence-directed antibody mapping of the proteolytic fragments have implicated putative extracellular domains in forming a DHP binding site. These regions were identified as a portion of the loop linking the fifth (S5) and sixth helix (S6) of the third domain, and also the sixth helix in repeats III and IV. Valdivia and Coronado (1990) have also reported that positively charged DHPs can interact with high affinity DHP binding site when applied to either side of a lipid bilayer into which T-tubule Ca^{2+} channels had been incorporated. Recently, it has been suggested that the two DHP binding sites that are also functionally coupled to VSCCs exist on opposite sides of the membrane (Valdivia and Coronado, 1990; Kass et al., 1991).

While there is still some controversy about the location of high affinity DHP binding sites, most available evidence suggests that the phenylakylamine binding site is accessible only from the intracellular surface. In reconstitution studies using planar bilayers, it was shown that D890, a charged derivative of verapamil, was effective only when applied to the intracellular-equivalent side (Affolter and Coronado, 1986). Recent studies have also shown that the peptide labelled by a photoactive derivative of verapamil lies between Glu-1349 and Trp-1391, a segment which lies on the presumed intracellular end of the sixth helix in the fourth homologous repeat (Striessing et al., 1990). Since the binding sites for DHPs and phenylalkylamines are thus proposed to be located at the opposite ends of transmembrane helix IVS6, it has been suggested that the allosteric interaction between these two sites may involve conformational changes induced by the movement of this IVS6 segment (Nakayama et al., 1991).

1.6.6 Modulation of Calcium Channels in Skeletal Muscle

So far, the best-established mechanism of Ca^{2+} channel modulation is the stimulatory effects of ß-adrenergic agonists mediated by cAMP-dependent kinase on the L-type cardiac Ca^{2+} channels. Occupation of the ß-adrenergic receptor by an agonist leads to the activation of a G-protein known as G_s which in turn, activates adenylate cyclase, hence producing cAMP. The increase in cAMP leads to the dissociation of the regulatory and catalytic subunits of the protein kinase A (PKA). The catalytic subunit of PKA phosphorylates the L-type Ca^{2+} channels and leads to an increase in their probability of opening (see Pelzer et al., 1990; Bers, 1991). L-type Ca^{2+} current in skeletal muscle is also augmented by intracellular application of cAMP and PKA (Arreola et al., 1987; Kokate et al., 1992). In biochemical studies it has been shown that the α_1 and ß subunits of skeletal Ca^{2+} channels are good substrates for PKA (Curtis and Catterall, 1985; Hosey et al., 1987). In functional studies, phosphorylation by PKA greatly increased the rate of $^{45}Ca^{2+}$ influx in reconstituted phospholipid vesicles containing purified Ca^{2+} channels. Similar observations have also been reported for the purified Ca^{2+} channels incorporated into planar phospholipid bilayers (Flockerzi et al., 1986; Hymel et al., 1988). In these preparations, the probability of channel opening was largely increased by PKA. In skeletal muscle, the T-type Ca^{2+} current is also augmented by ß-adrenergic/cAMP mediated stimulation (Arreola et al., 1987; Kokate et al., 1992).

Although phosphorylation of purified Ca^{2+} channels by PKC, CaM Kinase II and cGMP-kinase has been shown, their functional effects are not clearly established. In a recent interesting study on Ca^{2+} channels incorporated into lipid bilayers, Vilven and Coronado (1988) reported that IP_3 increased the nitrendipine-sensitive Ca^{2+} channel activity.

In addition to second-messenger mediated effects, direct regulation of skeletal Ca^{2+} channels by G proteins has also been identified (Yatani et al., 1987, 1988; Brown et al., 1989).

Activity of Ca^{2+} channels incorporated into lipid bilayers greatly increased by GTP-γ-S activated Gs or its activated α_s subunit (Yatani et al., 1988).

Thus, modulation of Ca^{2+} channels by various mechanisms appears to be more complex than was indicated by the initial studies on the regulation of cardiac Ca^{2+} channels by ß-adrenergic agonists.

1.6.7 Role of Ca^{2+} Channels in Excitation-Contraction Coupling

It has been well established that the entry of Ca^{2+} through voltage dependent Ca^{2+} channels plays an essential role in e-c coupling in cardiac and smooth muscle (Fleckenstein and Fleckenstein-Grün, 1989; Bers, 1991). In skeletal muscle, however, contractile force during twitches appears to be independent of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Armstrong et al., 1972; Frank, 1982; Lutgau, 1986) and the Ca^{2+} needed to activate contractile proteins comes mainly, if not entirely, from stores in SR. Voltage-sensitive Ca^{2+} channels are mainly concentrated in the T-tubules and due to their slow activation time course (with 100-200 ms peak time) they do not have enough time to open during an action potential spike that lasts only 2 to 2.5 msec (Beaty and Stefani, 1987). Fast-activated Ca^{2+} channels (with an activation time constant of 5 msec), however, can be activated during a single twitch (Cota and Stefani, 1986). A small $^{45}Ca^{2+}$ influx elicited by an action potential (0.2-1.3 pmol/cm^2; Bianchi and Shanes, 1959; Curtis, 1966) has been attributed to activation of these fast Ca^{2+} channels during a twitch (Cota and Stefani, 1986). On the other hand, in many studies it has been shown that slow Ca^{2+} channels can open during maintained contractures such as high K^+ induced contractures and voltage-clamp conditions (Frank, 1960, 1984; Chirandini and Stefani, 1973; Cota and Stefani, 1982; Ildefonse et al., 1985). Furthermore, it was also shown that high K^+ induced contractures can be blocked by various Ca^{2+} channel antagonists and in Ca^{2+} free solutions (Frank, 1958, 1984; Chirandini and Stefani, 1973) without blocking the twitch.

2. STATEMENT OF THE PROBLEM

In electrophysiological studies, during maintained depolarizations, the presence of a large Ca^{2+} inward current passing through L-type VSCCs located in T-tubules has been well established (Beaty et al., 1987). In biochemical studies, it has also been shown that skeletal muscle T-tubule membranes are the richest known source of DHP binding proteins (Fosset et al., 1983; Dunn, 1989). On the other hand, the functional roles of these channels have yet to be established (Catterall, 1991). Unlike cardiac muscle and most smooth muscle, in which calcium influx can initiate contraction, calcium entering skeletal muscle cells from the exterior through voltage-gated calcium channels is not required for initiating excitation-contraction coupling during twitch type mechanical responses (Armstrong et al., 1972; Frank, 1982a,b).

In addition to the twitches, various ways of eliciting mechanical responses in skeletal muscle fibres have been described (Zachar, 1971). In 1930, Gasser indicated the important distinction between contractions initiated by action potentials and contractures referring to muscle shortening or tension produced by other means. From this point of view, twitches and tetani are examples of contractions. On the other hand, high K^+, voltage-clamping or ACh induced mechanical responses can be considered as contractures.

A distinctive property of contractions such as twitch and tetanic responses is their dependence on the development of an action potential (Guyton, 1986). Since the development of action potentials and its inward spread into the T-tubule system is a part of the physiological process of e-c coupling (Constantin, 1970), contraction type of mechanical responses can be considered as relatively physiological responses. Furthermore, motor neurons innervating the muscle fibres produce a burst of action potentials that can last a few seconds at different frequencies (Kernell, 1965a,b). The repetitive firing of motor neurons brings about a mechanical response, tetanic contraction, that is used to produce force development during daily activities

of muscles. Hence, during an attempt to investigate the functional role of VSCCs in fast, twitch fibres of frog skeletal muscle, tetanic co. ..actions can be considered as a type of response that is quite appropriate to study for this purpose.

Although the requirement for the influx of extracellular Ca^{2+} through VSCCs during high K-induced contractures and long-lasting voltage clamp pulses, is well established (Frank, 1984, Ildefonse et al., 1985), the source of Ca^{2+} during tetanic contractions has not been studied in detail. Since, during physiological functioning of skeletal muscle fibres *in vivo*, single action potentials do not normally occur but action potentials occur in bursts, it was our hypothesis that the functional role for these VSCCs is to permit the entry of extracellular Ca^{2+} ions during repetitive action potential trains and that these Ca^{2+} ions are required for the muscle fibres to produce their maintained contractions. For this reason the effects of Ca^{2+}-free Ringer's solutions and organic Ca^{2+} channel blockers were studied to evaluate the functional roles of the VSCCs in tetanic responses.

Since another property of repetitive action potentials is the presence of late after-potentials (LAPs), we also conducted studies to investigate if VSCCs can permeate extracellular Ca^{2+} in the range of LAPs. Due to the difficulties of assessing the membrane potential deep in isolated T-tubules, we have employed the T-tubule membrane vesicles to determine whether Ca^{2+} transport occurs in the range of LAPs recorded during tetanic contractions. Ca^{2+} fluxes in T-tubule membranes were also characterized with respect to their pharmacological properties.

3. MATERIALS AND METHODS

'Though this be madness, yet there is

method in't'

Shakespeare: Hamlet, II - ii

3.1 Materials

3.1.1 Solutions

Ringer's solution prepared in distilled and deionized water (Whatmann deionizer) was used as the physiological solution in all experiments using intact muscle fibres. The composition of the bicarbonate-buffered Ringer's solution (normal Ringer's) was (in mM): NaCl, 111.8; KCl, 2.47; $CaCl_2$, 1.08; $NaHCO_3$, 2.38; NaH_2PO_4, 0.087; Dextrose 11.1; and d-tubocurarine 0.1 mg/ml. Tubocurarine has been shown to block specifically the neurotransmitter action of acetylcholine and was employed to eliminate any possible neuromuscular effect.

- In experiments requiring the use of a Ca^{2+}-free solution, $CaCl_2$ was omitted from the Ringer's (normal) solution. It was previously reported that Ringer's solution prepared in this way contains less than $10 \pm 1.3 \times 10^{-6}$ M Ca^{2+} (Frank, 1982). In order to observe the effects of reduced Ca^{2+} in a solution in which Ca^{2+} is more efficiently eliminated, the Ca^{2+} chelator, EDTA at concentrations between 10^{-5} to 5×10^{-3} M was used in Ringer's solution.

All solutions were prepared fresh for each experiment. The osmolarity of the solution was 230-235 mOsm/kg (Osmometer, Wescor 5500) and the pH was adjusted at room temperature to be between 7.2 and 7.4.

3.1.2 Drugs

Drugs used in this study were:

a) Nitrendipine - Miles Laboratories Inc., New Haven, CT

b) Nifedipine - Sigma Chemical Company

c) Verapamil - Knoll AG, Ludwigshafen

d) (±) Bay K8644 - Miles Laboratories Inc., New Haven, CT.

e) (+) PN 200-110 - Sandoz Ltd., Basel, Switzerland

f) (−) PN 200-110 - Sandoz Ltd., Basel, Switzerland

g) (+)-SDZ 202-791 - Sandoz Ltd., Basel, Switzerland

h) (−)-SDZ 202-791 - Sandoz Ltd., Basel, Switzerland

Drug solutions were routinely prepared by dissolving the required amount of the substance directly in Ringer's solution. Exceptions to the general procedure were as follows:

- In experiments with intact muscle fibres, nitrendipine was initially dissolved in purified dimethylsulfoxide[1] (DMSO) prior to further dilution with Ringer's solution. The maximum concentration of DMSO used was 2% by volume and the vehicle was included in all of the Ringer's solutions used in these particular experiments. DMSO at these concentrations produced a rapidly developed, constant and stable inhibition of twitch amplitude by about 5 to 15% over 3-4 hr in control experiments but it produced no change in the tetanic responses. Other than this slight decrease at the maximum amplitude of twitch responses, DMSO has no effect on either mechanical or electrical properties of skeletal muscle fibres. Somewhat similar findings for the effect of DMSO on other muscular tissues including smooth and cardiac muscle, have also been observed (personal communication with Dr. M. Wolowyk).
- In experiments with T-tubule membrane vesicles, all dihydropyridine derivatives were added from stock solutions in DMSO and, in order to minimize possible solvent effects, all samples, including controls, contained DMSO at the same final concentration (< 0.2%; Dunn, 1989).

[1]The compound was obtained through the kind courtesy of Dr. M. Wolowyk.

3.1.3 Radioactive Compounds and Liquid Scintillation Fluid:

The isotope of Ca^{2+} used was ^{45}Ca obtained from ICN.

[^{3}H] Nitrendipine and (+) - [^{3}H] PN200-110 were supplied from Du Pont- New England Nuclear.

The scintillation fluid used to measure ß-emission from $^{45}Ca^{2+}$, [^{3}H] Nitrendipine and (+) [^{3}H]PN200-110 was ACS, a commercial xylene-surfactant based aqueous counting scintillant from Amersham.

3.2 Methods

3.2.1 Tension Studies

3.2.1.1 Muscle Preparation

The extensor longus digiti IV (toe) muscles of the frog *Rana pipiens* were used in all experiments involving tension studies. Each frog was decapitated and pithed and the muscle dissected and removed. After removal, the muscle with each of its tendons tied to a silk thread was placed in a glass petri dish containing the appropriate physiological solution. The silk threads were then wrapped around two plastic pegs which were stuck to the inner surface of the dish close to its circumference and at diametrically opposite ends. This helped in positioning the muscle in the petri dish and facilitated further dissection. Care was taken not to stretch the muscle excessively. With the aid of a dissection microscope (Wild-Heerbrugg, Switzerland) the muscle was freed from connective tissue and fascial membranes. All muscles were allowed to equilibrate for a period of 30-45 min following dissection in the physiologic solution before an experiment was commenced.

3.2.1.2 Muscle Chambers

The muscle chambers used for tension recordings were constructed from glass or plastic barrels of syringes of 3 or 5 ml capacities. The toe muscle was mounted vertically in the chamber between a glass hook and a strain gauge. The proximal end of the muscle was secured to the glass hook near the bottom of the bath and the distal end was attached to the arm of the strain gauge positioned above the bath, by means of the silk threads. The position of the strain gauge was adjusted using a micromanipulator. The solution in the chamber was changed by draining at the bottom and by introducing fresh solution at the top. The resting tension of the muscle was adjusted so that muscle just failed to remain in a vertical position when the bath solution drained and to return to a vertical position when the bath was refilled with solution. This length is presumably near or at the resting length because the largest twitches were produced at this muscle length.

3.2.1.3 Stimulation Parameters

The toe muscles were stimulated by means of two ring electrodes of platinum situated near the top and bottom of the solution in muscle chamber. For single twitch responses, supramaximal rectangular pulses, 1.5 msec in duration were used. For tetanic responses, the muscles were stimulated with 1.5 msec supramaximal rectangular pulses at a frequency of 100 Hz for 2 sec once every 5 to 15 min unless otherwise indicated. Tissues remaind viable for up to 3 hours.

3.2.1.4 Tension Recording

Isometric tension was recorded by means of the strain gauge whose active elements consisted of two pixie transducers (Endevco model 8121A) in a Wheatstone bridge configuration.

The transducer outputs were digitized and recorded using a Tekmar Lab Master A/D converter board connected to a Compaq portable computer. The records were stored on floppy disks during the experiment. These records were later analyzed using a Compaq Deskpro 286 and the responses were drawn on a Roland DGPR-1212A printer. Responses were recorded with 1000 points per sweep at all sweep speeds. Programs for collecting, transmitting, storing and analyzing data were written in Turbo Pascal by Dr. G.B. Frank.

3.2.1.5 General Experimental Protocols

The toe muscles were excised and mounted as described above. In experiments involving the recordings of twitches and tetanic responses, the Ringer's solution bathing the muscle was continuously bubbled with a gas mixture of 99.5% O_2 and 0.5% CO_2. By employing this particular gas mixture, the pH of the Ringer's solution remained at approximately 7.2. The solution bathing the muscle, with or without drug application, was changed usually every 30 min or less during an experiment. Under these conditions, the mechanical responses for twitches and tetanic responses, elicited with the stimulation parameters described above, were recorded every 5 to 10 min. If the tension height of the control twitches and the area under tetanic force x time remained steady for 30 to 45 min, the tissue was considered suitable for an experiment. After obtaining the 'control' twitches and then 'control' tetanic contractions, the responses were recorded in the presence of the drug and they were compared with the 'control' responses obtained earlier.

3.2.1.6 Analysis

Measurements of the maximum twitch and tetanus heights, and the tension x time area were taken from recordings made on printer paper. Since it was only possible to obtain one

measurement of particular drug per tissue, the effects at various drug concentrations were normalized and arrayed from different experiments to construct the dose-response curves for different drug applications. In depicting the data, the arithmetic mean of different experiments was calculated and plotted with standard errors of the mean (SEMs) presented as vertical bars.

3.2.2 Electrophysiological Studies

Experiments were performed at room temperature (20°C) with the extensor longus digiti IV (toe) muscle isolated from the leopard frog, *Rana pipiens*. The following experimental procedures used in this study have been published (Oz and Frank, 1991, 1992).

3.2.2.1 Muscle Preparation

Although our goal was to obtain intracellular recordings of repetitive action potentials in relatively physiological conditions, these recordings are hampered by the mechanical artifacts produced by the movement of the muscle fibres. Thus far, several different methods have been used to reduce the mechanical artifacts. Movable microelectrodes have been developed, but their preparation and their successful application is difficult (Vaughan Williams, 1959). Other methods include physically stretching the muscle, sufficient to eliminate the overlap of the A and I bands and thereby to prevent movement (Fatt and Katz, 1951) or to immerse skeletal muscle in a hypertonic solution which also greatly reduces movement (Hodgkin and Horowicz, 1957; Howarth, 1957). However, both of the latter methods may be ineffective in completely stopping muscle movement during tetanic stimuli (Hodgkin and Horowicz, 1957; Lannergren and North, 1973), and in addition they produce volume changes in the T-tubules (Birks and Davey, 1972). Glycerol-removal treatment of muscle fibres to block the surface openings of the T-tubules also has been proposed for the prevention of movement artifacts, but glycerol treatment causes many

deleterious effects on T-tubule morphology (Howell, 1957), and a complete depression of the after potentials of muscle fibres (Gage and Eisenberg, 1969b).

In order to study the magnitude and time course of late after potentials (LAPs) in frog toe muscle twitch fibres under more physiological conditions and in normal, Na^+-Ringer solutions, we modified previously reported methods (Fatt and Katz, 1951; Stefani and Schmidt, 1972) and with the following techniques we were able to increase the duration of reliable recording during repetitive stimulation for much longer than possible with previous techniques. A glass rod of 3-4 mm thickness was mounted horizontally in a plexiglass bath (with a Sylgard® base) (Figure 5). The toe muscles were excised as described previously. One tendon of the toe muscle was attached to a micromanipulator, and then wrapped around and stretched on the glass rod. The other end of the muscle was attached to a second micromanipulator, and the muscle was stretched to about 120% of its rest length. By using the micromanipulators, it was possible to adjust the muscle length from both ends to reduce the muscle movements.

The use of a glass rod instead of polyethylene or plastic rods used in earlier studies, reduced the friction between muscle membrane and the rod, and thereby significantly reduced the stretch required for successful recording. The recording bath was continuously perfused with Na^+-Ringer solution and bubbled with a gas mixture of 99.5% O_2 and 0.5% CO_2 during the recording and for 10 min prior to the start of the recording. With this treatment i.e. less stretching and perfusion with an oxygenated solution, the viability of muscle fibres increased up to a few hours (Oz and Frank, 1992).

Using conventional intracellular recording techniques, resting and action potentials of twitch fibres were found to be no different from those measured in frog sartorious twitch muscle fibres in the horizontal placements (Frank, 1957). A representative recording of a repetitive action potential train and LAP is been shown in Figure 6. The time course of action potential

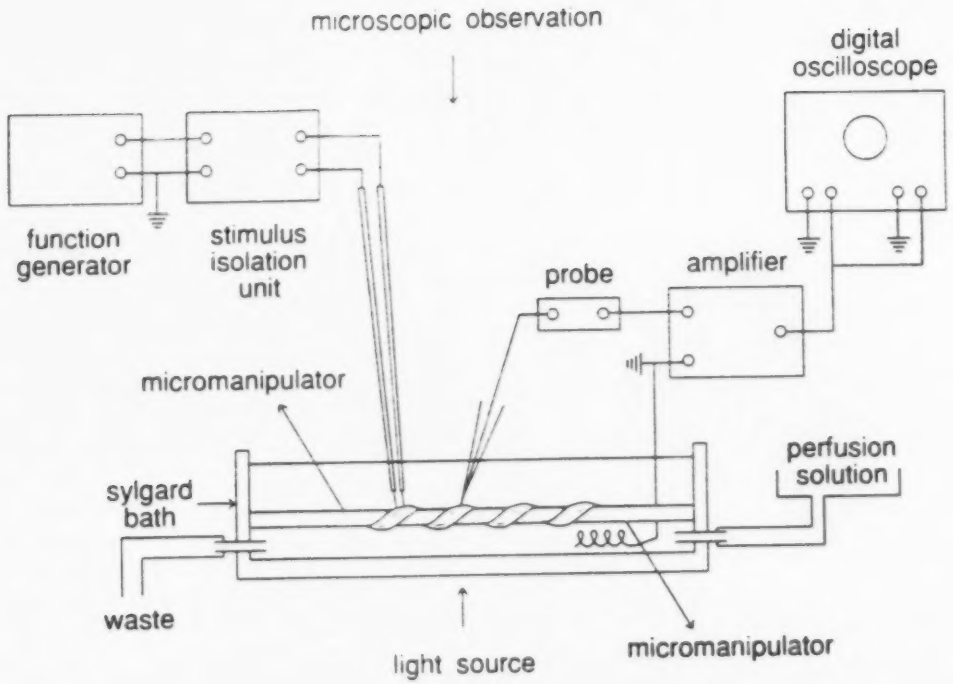


FIGURE 5: The modified arrangement of experimental setup for recording repetitive trains of action potentials with an intracellular microelectrode. Frog toe muscle was wrapped on a glass rod and the length of muscle was adjusted by means of micromanipulators from both sides. The recording bath was continuously perfused with the appropriate solution.

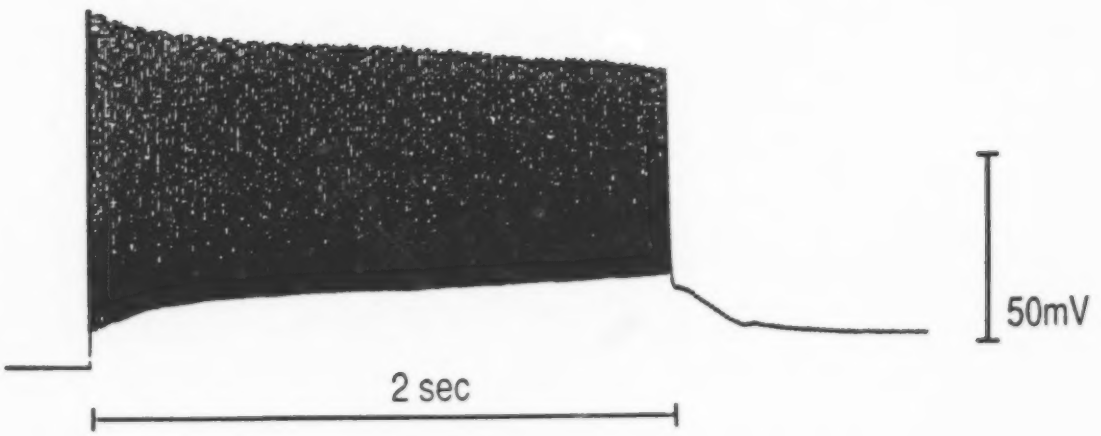


FIGURE 6: Typical intracellular recording during 100 Hz stimulation frequency for 2 seconds.

is clearly seen. The oscilloscope used had a sweep of 4000 points at all sweep speeds. For 0.5 sec stimulus trains the sweep duration was 800 msec; thus for the action potential spikes, which lasted about 2.5 msec, there were about 12.5 points, which was sufficient to record the peaks. At this sweep speed there were about 0.75 points for each artifact produced by the 0.15 msec stimuli. With 1 sec trains there were only 5 points for each spike and so the spike height seems to wax and wane as the maximum voltage point recorded on succeeding spikes approaches and departs from the spike maximum voltage (an aliasing effect). With 2 sec trains there were only about 2.5 points per action potential spike and the aliasing effect became more pronounced. Also with 2 sec trains there were only about 0.15 points per stimulus artifact and so most stimulus artifacts are missed (i.e. not recorded at all). The amplitude of LAPs at the end of the 2 sec pulse duration can easily be measured under relatively physiological conditions. Resting membrane potentials of muscle fibres before and after repetitive stimulations did not change, and LAPs slowly declined back to the previous resting potential values.

3.2.2.2 Stimulation and Recording System

A small proportion of the muscle fibres were electrically stimulated by an extracellular, bipolar platinum-filled pore electrode placed on the surface of the muscle 5 to 10 mm from the recording electrode. The bipolar stimulating electrode was constructed with 0.4 mm platinum wires placed in polyethylene tubing and cemented together so that the ends of the wires, which rested on the surface of the muscle, were 1.5 mm apart. The muscle fibres were stimulated with supramaximal pulses delivered through a stimulus isolator unit (WP Instruments, model 305). The stimulating pulse consisted of either a single stimulus or a train of square waves of 0.15 msec duration and 10 msec apart. Resting membrane and action potentials of the frog toe muscle fibres were recorded intracellularly using conventional glass capillary microelectrodes (10-40 M

Ω resistance) filled with 3 M KCl. The glass microelectrode was held in a microelectrode holder, which was connected through a probe to the main amplifier (WP Instruments, model MA4). The output from the amplifier was displayed on a model 3091 Nicolet digital oscilloscope. The reference electrode consisted of a chloride silver wire, formed into a spiral, and placed in the solution bathing the muscle. The recordings were made with 4000 points per sweep at all sweep speeds. These records were stored on floppy disks and later analyzed, and pictures of action potentials were drawn using a Compaq Deskpro 286 computer and a Roland DG PR-1212A printer. All programs for transmitting, storing and analyzing data were written in Turbo Pascal by Dr. G.B. Frank.

3.2.2.3 Recording Electrodes

Recording electrodes were made from open-ended glass capillaries (Fisher Scientific Co.) having 1.5-2 mm outside diameter. The microelectrodes were drawn by a glass microelectrode puller (PN-3 Narishige Scientific Instrument Lab Ltd., Japan). The settings of the microelectrode puller were adjusted such that the tip length of the electrodes measured approximately 9-10 mm. These microelectrodes were then mounted on microscope slides with a rubber band wrapped around them and placed in a Coplin straining jar with their tips downward. The Coplin jar was then filled with methanol and placed in a vacuum desiccator. The methanol was allowed to boil for about 5 to 10 min by reducing the pressure in the desiccator. With this procedure, almost all the electrodes were completely filled with methanol. The methanol was subsequently replaced by distilled water and the jar was left overnight at atmospheric pressure. Finally, the microelectrodes were placed in 3 M KCl solution and left there for a minimum of 24 hours before use. The resistance of the microelectrodes was measured by a solid state volt-ohm-microamp meter (Danameter, Dana Lab. Inc., Irvine, California) and only those electrodes

having resistances between 10 and 40 MΩ were selected for use.

3.2.2.4 General Experimental Protocols

Electrical events were recorded from (usually) 2-4 surface fibres over a short time period (about 5 min overall). Control recordings were taken after a 10-15 min continuous perfusion with Ringer's solution bubbled with a gas mixture of 99.5% O_2 + 0.5% CO_2 during the equilibration period.

After the control readings were obtained, the tissue bathing medium was replaced with a solution with a drug and the tissue was continuously perfused with Ringer's solution containing the same drug. Traces of the electrical events were obtained at frequent time intervals, viz., 3-6, 9-12, 14-16 and 25-30 min following exposure to the drug.

3.2.2.5 Analysis

The effect of any given concentration of a drug on late after-potentials was evaluated by comparing the maximum amplitudes of the late after-potentials. For this purpose, firstly we calculated the mean of the maximum amplitudes of LAPs in control conditions and in the presence of drug for each muscle. After these statistical calculations were done, a paired t-test was performed. A confidence level of 95% (P : 0.05) was chosen in our statistical evaluation.

3.2.3 Ca^{2+} Efflux Studies in T-Tubule Membrane Vesicles

3.2.3.1 Preparation of Microsomal Vesicles

Microsomal membranes were prepared from the back and hind leg muscles of New Zealand White rabbits by a modification of methods described earlier (Fernandez et al., 1980). In order to prevent the degradation of proteins by proteolytic enzymes, all procedures were

carried out at 0-4°C. The excised muscle parts were cut into small pieces and added into 4 volumes of homogenization buffer composed of 20 mM Tris-HCl, 0.3 M sucrose, 5 mM EDTA, 1.2 mM EGTA and the protease inhibitors, 1 mM iodoacetamide, 0.5 mM phenylmethyl sulfonyl fluoride, 0.1 mM benzamide and 1 μM pepstatin A. 0.02% NaN_3 was included as antibacterial preservative. The pH was adjusted to 7.4 for all solutions used. The muscle suspension was homogenized using five 20-sec high speed bursts of a Waring blender. The microsomes were isolated by differential sedimentation, removing mitochondria and nuclei by centrifugation for 20 min at 4000 x g_{max} (5000 rpm in a Sorvall GSA rotor). Following this centrifugation, the pellet was discarded and the supernatant was recentrifuged for 20 min at 10,500 x g_{max} (8000 rpm in a GSA rotor). The supernatant was filtered through six layers of cheesecloth to remove remnants of pellet and fat particles, and solid KCl was added to a final concentration of 0.5 M to solubilize contractile proteins in the supernatant. This mixture was stirred for 30 min at 4°C with a magnetic stirrer and then centrifuged for 45 min at 186,000 g_{max} (40,000 rpm in a 45 Ti Beckman Rotor). The microsomal pellets were resuspended in homogenization buffer using two or three 20-sec bursts of a Virtis 45 Homogenizer at the setting of 50. The resuspended pellet was washed twice by centrifugation in a 45 Ti (Beckman) rotor at 40,000 rpm.

The microsomal membranes were finally resuspended in Tris buffer containing 20 mM Tris-HCl, pH:7.4; 15% (w/v) sucrose. The microsomal membranes were either used immediately for further studies or frozen in liquid nitrogen and stored in a refrigerator at -80°C. Binding activities for (+)-[^{3}H]PN200-110 in these membrane preparations were 4-10 pmol/mg of protein.

3.2.3.2 Preparation of Transverse Tubule Membranes

Discontinuous sucrose gradient centrifugation was used for the preparation of T-tubule

membrane vesicles. Three layers of 10 ml volumes of 35% (w/v), 27.5% (w/v), and 25% (w/v) sucrose in 20 mM Tris-HCl, pH:7.4 were used to form discontinuous sucrose gradients. The microsomal membranes in 20 mM Tris-HCl, 15% (w/v) sucrose pH:7.4 were loaded on top of three discontinuous sucrose gradients (6 ml membranes/gradient). After centrifugation in a Beckman SW 28 rotor at 22,000 rpm for 16 hours at 4 °C, bands were found on top of the 25% layer and at the 27.5%/35% interface, with a large pellet at the bottom of the tube. The lightest band contained T-tubules essentially free of SR contamination. Some T-tubules and mostly light SR vesicles were found in the 27.5%/35% sucrose interface. The rest of the SR, mainly heavy SR vesicles, was found in the pellet (Fernandez, 1980). The floating layer of 3 to 4 ml was discarded. Only the lightest fraction from the 15%/25% sucrose interface was used in all experiments described in these studies. Bands were collected by aspiration. The sucrose contained in these bands was diluted by dropwise addition of 20 mM Tris-HCl to these membranes while continuously stirring on ice. Suspended T-tubule membranes were collected by centrifugation for 30 min at 186,000 x g_{max}, and the pellets were resuspended in the desired buffer using two 20-sec bursts of the Virtis 45 homogenizer at setting 50. In order to equilibrate in either low K^+ (10 mM Hepes-Tris, pH 7.4, 145 mM choline chloride, 5 mM potassium gluconate) or high K^+ (10 mM Hepes-Tris, pH 7.4, 150 mM potassium gluconate) buffer, the membranes were diluted in a large volume of the appropriate buffer and washed at least twice by centrifugation and resuspension. Following final resuspension in 0.2-0.5 ml of buffer, the membranes were frozen in liquid nitrogen and stored at -86 °C until required, or thawed, and stored on ice prior to use. This freeze-thaw cycle appears to be sufficient for the equilibration of intra- and extravesicular ions, since similar results have been obtained using vesicle preparations that had been equilibrated by prolonged incubation (16-20 h) at 4 °C in the appropriate buffer (Dunn, 1989). Protein concentrations was assayed using Bio-Rad protein assay

kit.

Characterization of T-tubule membranes isolated using this method has been described in a number of studies (Hidalgo et al., 1986; Dunn, 1989). In rabbit T-tubule membranes prepared by this method, Dunn (1989) has found that the approximately 90% of vesicles were sealed. In various studies it has been well established that the T-tubule membrane preparations consist mainly of sealed membrane vesicles that are oriented in such a way that the cytosolic face of the T-tubules forms the outside of the vesicle, and the luminal surface of the T-tubules *in situ* comprises the intravesicular wall of the vesicle membrane. Since the term, inside out has been extensively used to define this form of vesicles (Rosenblatt et al., 1981; Hidalgo et al., 1983; Hidalgo et al., 1986), our laboratory has followed this original terminology (Dunn, 1989; Bhat et al., 1992; Oz et al., 1992).

3.2.3.3 Equilibrium Binding of [^{3}H]Nitrendipine and (+)-[^{3}H]PN200-110

The binding activity of [^{3}H]nitrendipine and (+)-[^{3}H]PN200-110 was measured in filtration assays that were carried out under subdued lighting to minimize ligand photolysis. Aliquots of T-tubule membranes (400 μl) were added to different concentrations of radiolabelled ligand to give a final protein concentration of 0.05 mg/ml in a total volume of 0.8 ml assay buffer (25 mM Hepes-Tris, pH:7.4, 1 mM $CaCl_2$, 0.02% NaN_3). After the samples were prepared, they were covered with aluminum foil and incubated 60 min at room temperature. Following this incubation period, 0.4 ml aliquots of each sample were removed rapidly and filtered under vacuum through Whatman GF/C filters using a Hoefer filtration manifold. The filters were immediately washed with two 5-ml aliquots of ice-cold buffer composed of 25 mM Hepes-Tris, pH:7.4, 1 mM $CaCl_2$, 0.02% NaN_3. After drying and addition of 5 ml of ACS scintillation fluid, the filters were counted for ^{3}H. Duplicate 50-μl samples of the incubation

mixtures were also counted directly for estimations of total ligand concentration. Nonspecific binding of radioactively labelled ligand was estimated from parallel measurement of binding in the presence of 1 μM unlabelled ligand. All experiments were carried out at room temperature (23 $\pm$ 2 °C).

3.2.3.4 $^{45}Ca^{2+}$ Efflux Studies

In all flux experiments, membrane vesicles were first equilibrated in low K^+ buffer as described above. The vesicles (approximately 0.4 mg/ml were loaded with $^{45}Ca^{2+}$ by the addition of one-half volume of isotopically diluted $^{45}CaCl_2$ solution in the same buffer to give a final concentration of 5 mM total Ca^{2+} containing approximately 50 μCi/ml $^{45}Ca^{2+}$. The mixture was then subjected to two freeze-thaw cycles using liquid nitrogen as previously used to load impermeant molecules inside membrane vesicles (Moore and Raftery, 1980). The Ca^{2+}-loaded vesicles were kept on ice until use, which was usually within 3 h. In order to induce $^{45}Ca^{2+}$ efflux, a two-step filtration assay (Dunn, 1989) was used. In this experimental procedure, 25 μl of loaded membranes were first diluted with 975 μl of high K^+ buffer (10 mM Hepes-Tris, pH 7.4, 120 mM potassium gluconate, 30 mM choline chloride, 0.133 mM EGTA) containing 0.1 μM valinomycin. This first dilution is designed to mimic the resting state of the cell by generating an outside negative membrane potential of -80 mV and to reduce the extravesicular (corresponding to intracellular in an inside out vesicle) free Ca^{2+} to less than 100 nM. After 5 min incubation at room temperature, 0.9 ml was removed and applied to a GF/C filter which had been preequilibrated in the same buffer, mounted in a Hoefer filtration apparatus, and dried under vacuum. Excess buffer was removed from the sample under vacuum, and 1 ml of depolarizing buffer (10 mM Hepes-Tris pH 7.4, 54 mM potassium gluconate, 96 mM choline chloride, 0.133 mM EGTA, 0.1 mM valinomycin). Under these conditions, the membranes are depolarized to

an estimated -60 mV i.e. corresponding to the range of LAPs in intact muscle fibres. Efflux was allowed to continue on the filter for 20 sec prior to removal of extravesicular solution under vacuum and rapid washing with two 5 ml volumes of a "stop" solution (10 mM Hepes-Tris pH 7.4, 96 mM choline chloride, 54 mM potassium gluconate, 0.1 mM $LaCl_3$, 30 mM sucrose). Filters with their absorbed membrane vesicles were dried, extracted with 5 ml of ACS scintillation fluid, and counted for residual entrapped $^{45}Ca^{2+}$. In the flux studies, the appropriate drugs were incubated with $^{45}Ca^{2+}$-loaded vesicles for 30 min on ice prior to initiation of repolarization. All subsequent buffers contained the drug at the same concentration. Drugs were added from stock solutions in DMSO and, in order to minimize possible solvent effects, all samples, including controls, contained the drug at the same final concentration ($< 0.2\%$).

3.2.3.5 Data Analysis

The arithmetic means and standard errors of the mean of experimental results are presented as histograms. The number of experiments (n) carried out are indicated on the top of each histogram. Estimated membrane potentials quoted in the text are those predicted by the Nernst equation in the presence of potassium gradients across the membrane.

4. RESULTS

Science is always wrong. It never solves a problem without creating ten more.

- Bernard Shaw

4.1 Mechanical Responses

4.1.1 Effect of Calcium-Free Ringer's Solutions on Mechanical Responses

In Ca^{2+} free Ringer's solution, the contamination of Ca^{2+} through different sources has been reported (Frank, 1982b). Ca^{2+} free solutions that do not contain EDTA, can be contaminated with Ca^{2+} up to the concentrations of 10×10^{-6} M (Frank, 1982b). Since EDTA does not enter the muscle fibres (Frank, 1982a), it is used to chelate the contaminating Ca^{2+} and the Ca^{2+} that leaked out of the muscle fibres into the extracellular fluid in the intact toe muscles during stimulation. For this reason, EDTA at the concentrations varying between 10^{-5} to 5×10^{-3} M was added to Ca^{2+}-free Ringer's solutions.

After some preliminary observations, condi[illegible]ns were established in which the twitch was not reduced by the Ca^{2+}-free solution during a 10-min exposure period. This was done by adding only a very small concentration of EDTA to the Ca^{2+}-free Ringer's solutions.

As has been known for a long time, there is usually an initial phase of twitch potentiation, hyperexcitability and after discharges when the isolated muscle fibres are exposed to Ca^{2+}-free Ringer's solutions (Caputo and Gimenez, 1967; Sandow et al., 1975; Frank, 1982b). In our experiments, the initial effects of exposing whole toe muscle preparation (n: 52) to a Ca^{2+}-free Ringer's solution containing varying concentrations of EDTA (10^{-5} M to 5×10^{-3} M) were very similar to results obtained in isolated muscle fibres. These effects produce a greatly enlarged and prolonged mechanical response after a single stimulus (Fig. 7, B and C, twitches). Duration of this initial potentiation of twitch responses varied between 5 to 15 minutes with the concentration of EDTA used. These initial potentiating effects of Ca^{2+}-free solutions were replaced by the inhibition of twitch responses by 10 - 40 min depending on the concentrations of EDTA used in Ca^{2+}-free Ringer's solutions. The effects of increasing the EDTA concentration in the Ca^{2+}-free Ringer's solution on twitch tensions and tetanic areas were also studied. Experimental results

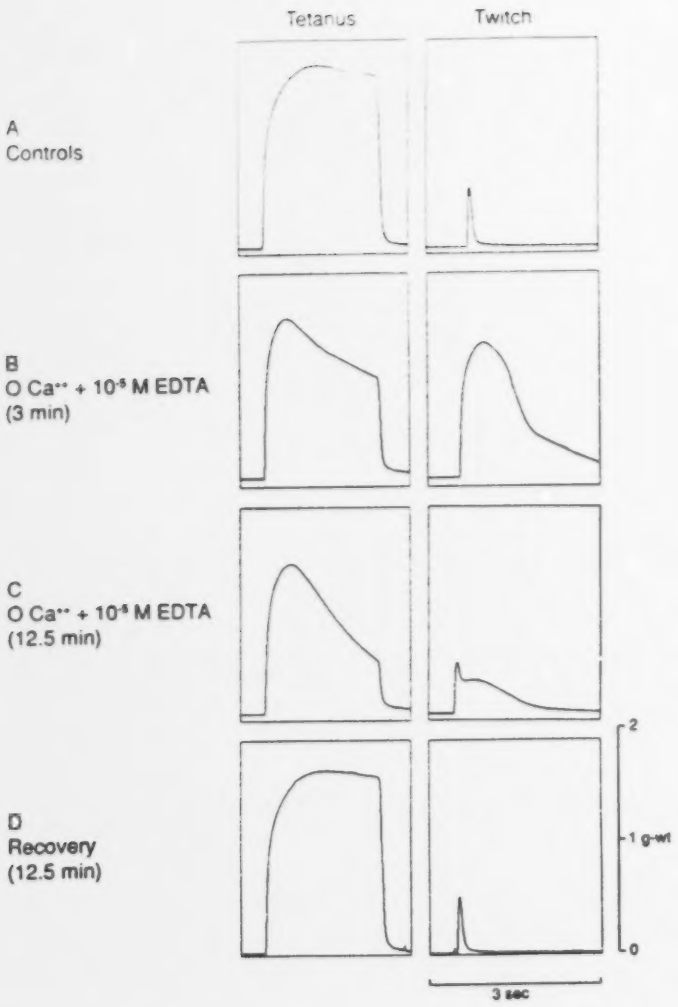


FIGURE 7: Effects of removing extracellular Ca^{2+} ions on twitches and tetani in an isolated frog toe muscle. The 0 Ca^{2+} solution contained a small amount of EDTA as indicated. Length of time the muscle was in the 0 Ca^{2+} solution is listed in B and C, and the time in Ringer's solution is listed in D. Stimulation frequency = 100 Hz.

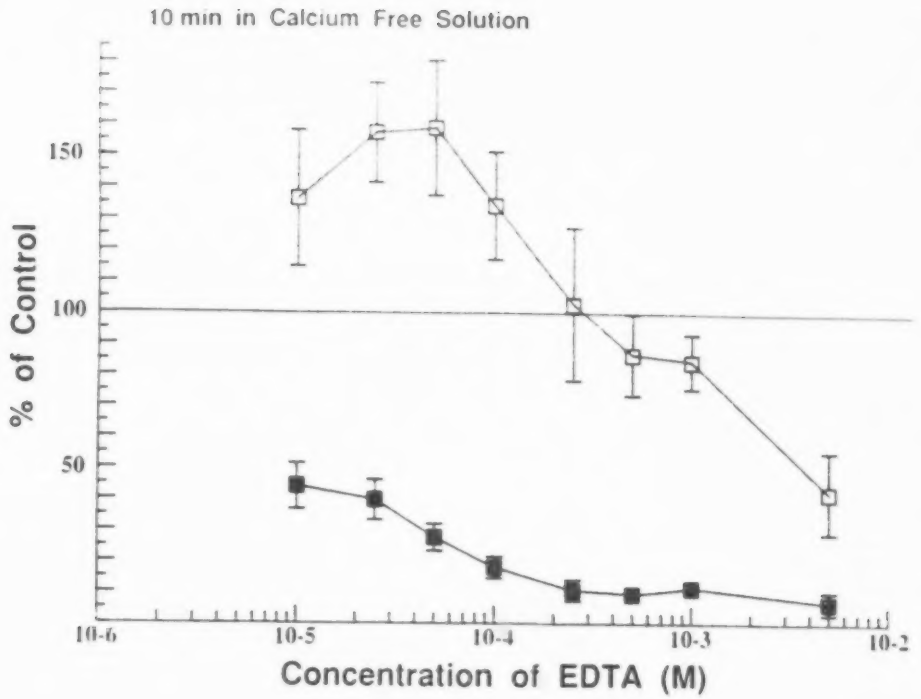


FIGURE 8: Effects of increasing the EDTA concentration on the twitches and tetani produced in frog toe muscles kept in the 0 Ca^{2+} solution for 10 min. The numbers of muscles (n) tested with increasing EDTA concentrations were 8, 8, 8, 8, 4, 8, 6 and 2. Means and standard errors of the mean are plotted. When the standard error is not shown it was smaller than the size of the symbol. Open symbols, twitch amplitude; filled symbols, tetanus area. Stimulation parameters of tetani = 100 Hz for 2 sec.

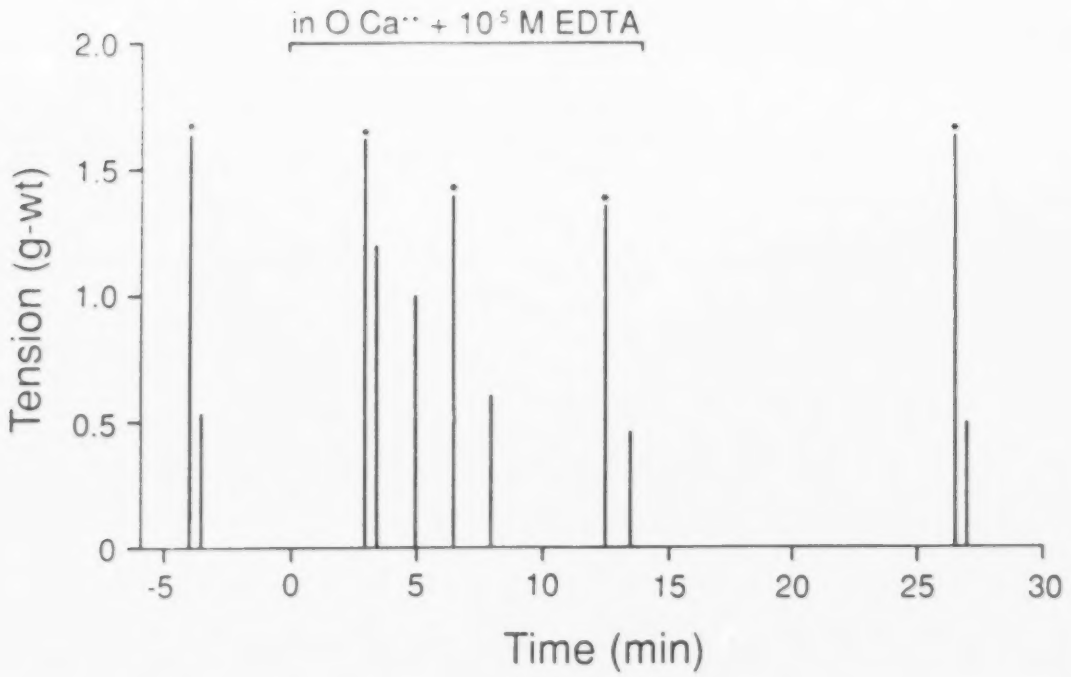


FIGURE 9: Effect of calcium-free Ringer's solution containing 10^{-5} M EDTA on the maximum tensions of twitches and tetani. The muscle was placed in the calcium-free solution at time 0. Tetani indicated by an * at the top of the line. All the responses obtained in a single experiment are shown. Stimulation parameters of tetani = 100 Hz for 2 sec.

collected from 52 different toe muscle fibres are shown in Figure 8. The 10 min exposure time was chosen because in isolated skeletal muscle fibre preparations twitches can often be maintained for up to 10 min in a Ca^{2+}-free solution containing 1 mM EDTA (Frank, 1982a,b). In our experiments it was found that the amplitudes of the responses to a single stimulus in a 10 min period were larger than control values at EDTA concentrations below 2.5 x 10^{-4} M (n: 36), and the twitches in 10 min were reduced by a great extent at higher EDTA concentrations (n: 16). The effects of Ca^{2+}-free Ringer's solution containing EDTA at these low concentrations recovered rapidly in 5 to 10 min. At higher EDTA concentrations recovery took 30 - 60 min.

Despite this increase in the size of the responses to single stimuli, the latter part of the tetanic responses at 100 Hz recorded $\leq$ 1 min earlier was depressed even though the maximum tension recorded near the start of the tetanus was not reduced. For this reason the effects on tetani were measured by obtaining the time x tension area of each tetanic response. In the results plotted in Figure 9, exposing a toe muscle to Ca^{2+}-free solution containing EDTA at a concentration of 10^{-5}M, had almost no effect on the maximum amplitudes of tetanic contractions, and there was an initial potentiation of twitches as described above. In summary, in contrast to the amplitude of single twitch responses that were larger than control in Ca^{2+}-free Ringer's solutions containing EDTA at concentrations below 2.5 x 10^{-4} M, the time x tension areas of tetanic responses were greatly reduced (50% to 10%; n: 16) (see Fig. 7 and 8). Although the maximum amplitudes of tetanic contractions did not change in this 10 min exposure time (Figure 9).

4.1.2 Effects of Nitrendipine on Twitches and Tetani

Nitrendipine, which can inhibit or block calcium ion uptake into skeletal muscle fibres without reducing twitches or producing hyperexcitability (Dretchen and Raines, 1984; Frank et

al., 1988; Frank, 1990), was used in experiments on toe muscles similar to those described above. There was a lack of effect of nitrendipine on maximum twitch or tetanic tensions at all concentrations (10^{-6} - 10^{-4} M; n: 20) used. For example, in the results plotted in Figure 10, exposing a toe muscle to 10^{-5} M nitrendipine for up to 70 min had essentially no effect on the maximum twitch or tetanic tensions. Nevertheless there was an obvious decrease in the area of the tetanic responses recorded within 1 min in the same experiment. Recordings of some of the tetanic responses obtained during this experiment are shown in Figure 11. Although there was no effect of nitrendipine at this concentration (10^{-5} M) on the maximum tensions of twitches, the area of tetanic response decreased to 65% (n: 4) of its control value. Depressant effects of nitrendipine on tetanic responses were normally reversed in 15 to 45 min. The very high concentration of nitrendipine (10^{-4} M) was an exception to this recovery time. At this high concentration recovery took more than an hour, or not at all.

The results obtained in 20 experiments of this type with nitrendipine concentrations from 10^{-6} to 10^{-4} M are plotted in Figure 12 and represented in Table II. Most of the twitch recordings were taken 1 min before tetanic contractions except in a few preliminary experiments where twitches were recorded after tetanic contractions. In these experiments control post tetanic twitches and post tetanic twitches measured during drug effect were compared in the same conditions, i.e. both after tetanus. In addition, no post tetanic potentiation (PTP) was observed in twitch responses measured after 2 sec lasting tetanic responses stimulated at 100 Hz. Similar results, indicating the lack of PTP in identical conditions, were also reported in other studies (Lopez, 1982; Fig. 6H, h). But for longer lasting contractions such as 10 to 50 sec, development of PTP was reported (Vergara et al., 1977). The only effect of nitrendipine on the twitch was to increase the maximum tension to up to 130% of control values. By contrast, nitrendipine greatly decreased the areas of the tetanic responses by up to 25% of control values. This

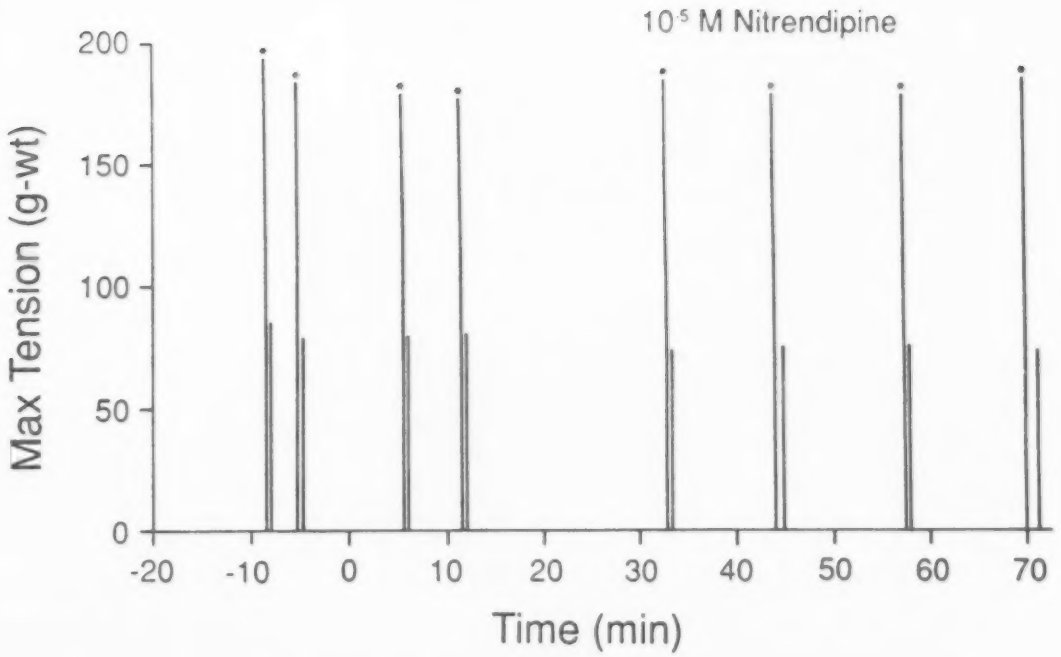


FIGURE 10: Lack of an effect of nitrendipine, at the indicated concentration, on the maximum tensions of twitches and tetani. The muscle was placed in the drug containing solution at time 0. Tetani indicated by an * at the top of the line. All the responses obtained in a single experiment are shown. Stimulation parameters of tetani = 100 Hz for 2 sec.

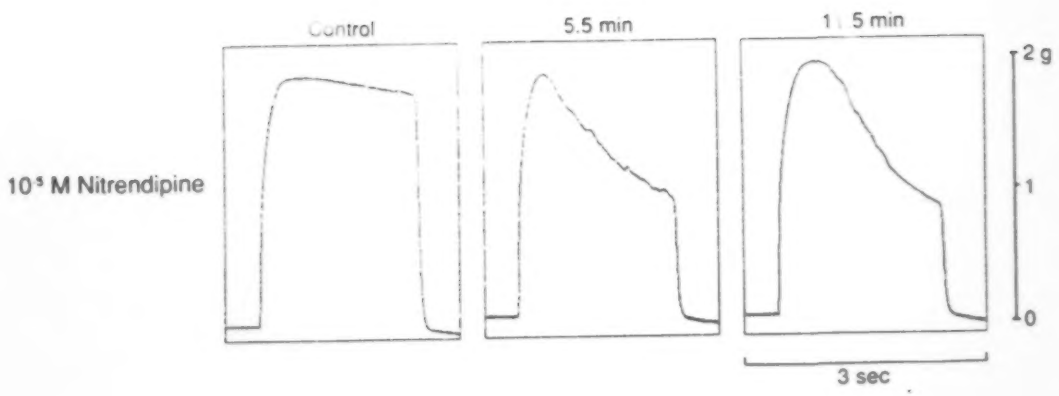


FIGURE 11: Recordings of some of the tetani obtained during the experiment plotted in Figure 15. The control tetanus was recorded just before exposing the muscle to nitrendipine and the first two tetani recorded in the drug solution are shown also. Stimulation frequency = 100 Hz.

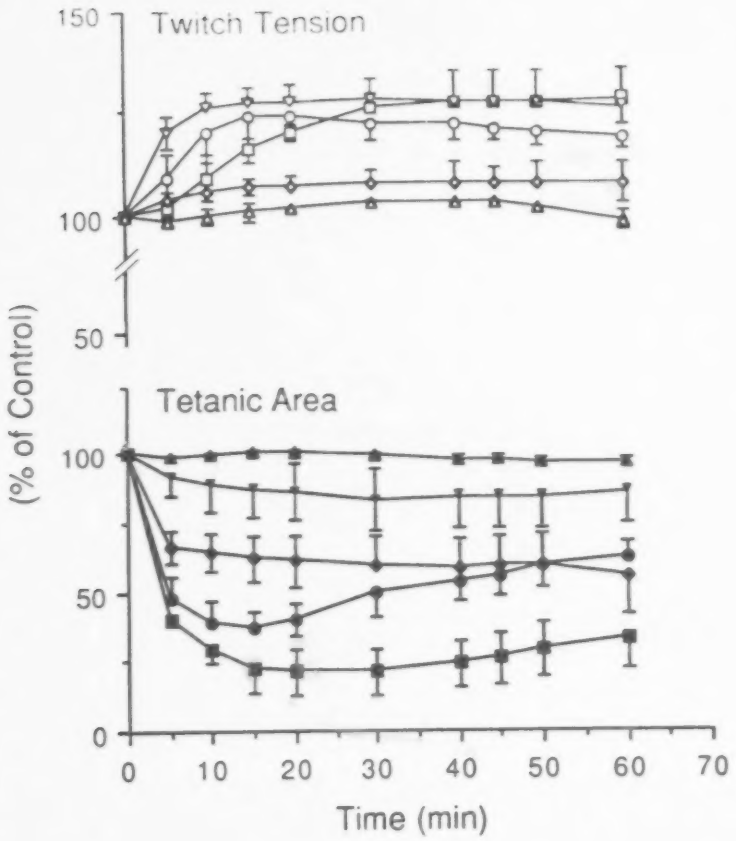


FIGURE 12: Effects over time of several nitrendipine concentrations on maximum twitch tensions (open symbols) and tetanus areas (filled symbols) in frog toe muscles. Points are the mean and standard error of the mean of at least 3 observations as noted below.. Standard error bars were omitted when they overlapped or when they were smaller than the size of the symbol. The nitrendipine concentrations (molar) and the number of experiments (n) were as follows: ▽, ▼: 10^{-6} (4); △, ▲ : 5 x 10^{-6} (3); ◇, ◆ : 10^{-5} (4); □, ■ : 5 x 10^{-5} (3); and ○, ● : 10^{-4} (6). 10^{-4} M was the highest drug concentration that could be obtained using 2 ml of DMSO per 100 ml of Ringer's solution. However, at this concentration the nitrendipine seemed to be forming colloidal particles over time, which probably explains why this concentration produced a smaller depression of the tetanus than the next lower concentration and why the tetanus depression starts to decrease after 15 min at this drug concentration. Stimulation parameters of tetani = 100 Hz.

TABLE II

Summary of Experimental Results with Different Concentrations of Nitrendipine

Concentr. of Nitrendipine (M)	(% of Control) Twitches / (% of Control) Tetani	5 min	10 min	15 min	30 min	45 min	60 min
10^{-6} n = 4	Twitches	119.75 ± 3.57	126.25 ± 4.54	119.50 ± 1.77	128.00 ± 5.53	125.75 ±	127.00 ± 4.95
	Tetani	91.75 ± 6.79	88.50 ± 10.12	98.50 ± 4.52	82.50 ± 11.33	86.50 ± 9.92	76.75 ± 13.55
5×10^{-6} n = 3	Twitches	99.33 ± 1.20	100.33 ± 2.67	101.67 ± 1.86	102.33 ± 1.20	100.50 ± 1.17	100.33 ± 2.73
	Tetani	98.33 ± 0.66	100.00 ± 1.53	100.67 ± 0.66	98.00 ± 1.16	96.00 ± 2.68	95.67 ± 2.19
10^{-5} n = 4	Twitches	104.00 ± 2.35	106.50 ± 2.18	107.25 ± 2.46	105.75 ± 4.39	107.63 ± 4.63	106.50 ± 5.75
	Tetani	65.50 ± 5.75	63.00 ± 8.01	61.00 ± 9.25	58.25 ± 11.65	60.13 ± 11.51	59.50 ± 14.18
5×10^{-5} n = 3	Twitches	101.33 ± 3.85	111.00 ± 6.57	117.33 ± 2.97	125.00 ± 4.17	126.84 ± 7.86	27.33 ± 8.85
	Tetani	40.00 ± 1.00	26.00 ± 6.43	21.33 ± 7.63	20.33 ± 7.87	30.17 ± 11.68	34.00 ± 11.86
10^{-4} n = 6	Twitches	108.50 ± 5.99	121.83 ± 5.97	125.17 ± 4.76	122.00 ± 4.07	121.50 ± 3.68	118.83 ± 3.51
	Tetani	38.5 ± 7.60	39.3 ± 7.21	38.2 ± 5.97	49.7 ± 9.20	55.34 ± 7.51	58.83 ± 6.46

depression was apparent by 5 min and approached a maximum by about 15 to 20 min. The decline in tetanic area over 20-min exposure time with the logarithm of increased nitrendipine concentration is plotted in Figure 13. The perfusion solution used in these experiments contained the solvent DMSO at a concentration of 0.28 M. DMSO at this concentration produced a rapidly developed, constant and stable inhibition on twitch amplitude of about 5 to 15% over 3 to 4 hr in control experiments, but it produced no change in the tetanic responses (see also Method section 3.1.3).

4.1.3 Effects of Verapamil on Twitches and Tetanic Responses

In these studies, experiments were conducted with 23 muscles using verapamil in the concentration range of 10^{-6} to 10^{-4} M. Experimental results showing the effects over time of several concentrations of verapamil (10^{-6} to 10^{-4} M) on maximum twitch tension and on the area under tetanic tension x time are plotted in Figure 14 and a summary of experimental data is tabulated in Table III. Unless high concentrations of verapamil ($\leq 10^{-4}$ M) were used, the maximum twitch amplitudes either did not change or slightly increased to 110% of control values by exposing the muscles to 10^{-6} M to 5 x 10^{-5} M verapamil (Fig. 14, top section).

On the other hand, verapamil decreased the area under 2 sec tetanic tension x time curves at 100 Hz stimulation frequency over the concentration range of 3 x 10^{-6} M to 10^{-4} M (Fig. 14, bottom section). The effect of verapamil over this range was large and the area under the tetanic curve was depressed by up to 96% of control values by 10^{-4} M verapamil (n = 4). With all the concentrations used, the effect of verapamil reached its equilibrium values by 5 to 10 min. The decline in area as a function of log verapamil concentration obtained using the data taken at 10 min is shown in Figure 15.

In a previous study with nitrendipine, the amplitude of the tetanus always declined

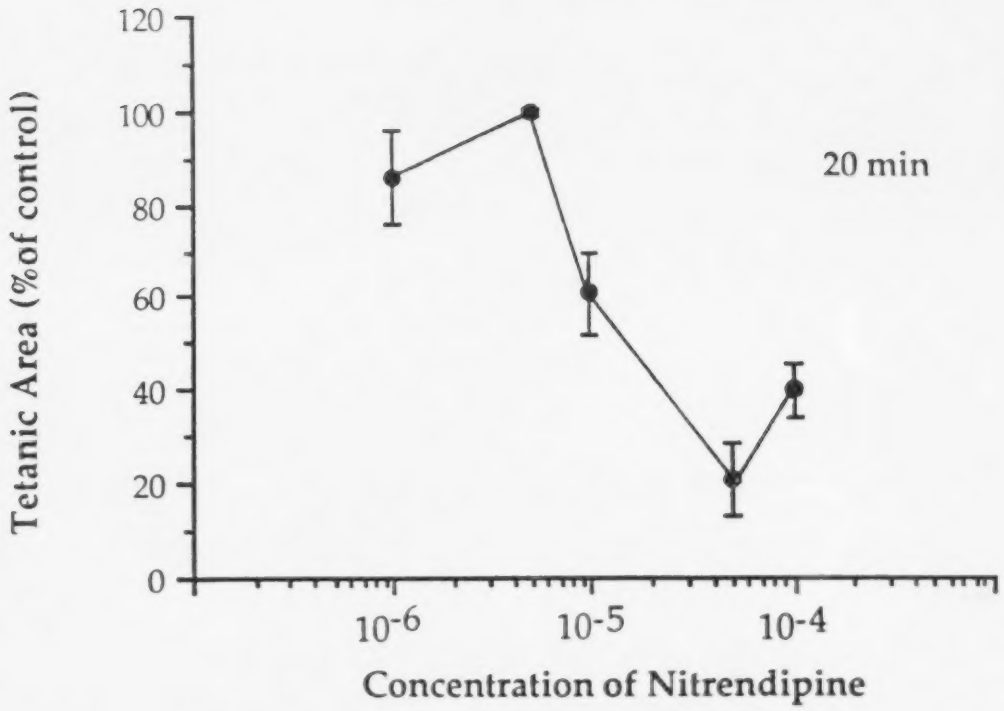


FIGURE 13: Effects of 20-min exposure to increasing concentrations of nitrendipine on tetanic areas (100 Hz for 2 sec). Means, standard errors and the numbers of experiments (n) at each concentration are given in the legend to Figure 12.

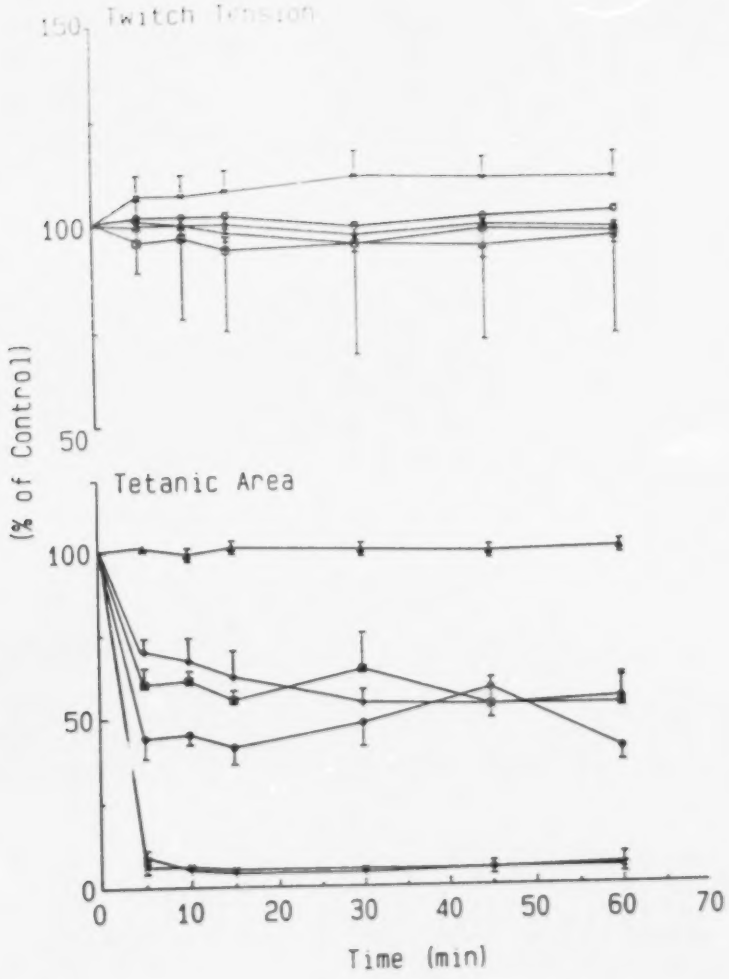


FIGURE 14: Effects over time of different verapamil concentrations on maximum twitch tension (top) and tetanus areas (bottom) in frog toe muscles. Points are the mean (± SEM) of at least 3 observations as noted below. Standard error bars were omitted when they overlapped or when they were smaller than the size of the symbol. The verapamil concentrations (molar) and the number of experiments (n) were as follows: △, ▲ : 10^{-6} (3); ◇, ◆ : 3 x 10^{-6} (4); □, ■ : 5 x 10^{-6} (3); ○, ● : 10^{-5} (3); X, X : 5 x 10^{-5} (3); and +, + : 10^{-4} (4). Stimulation parameters of tetani = 100 Hz for 2 sec.

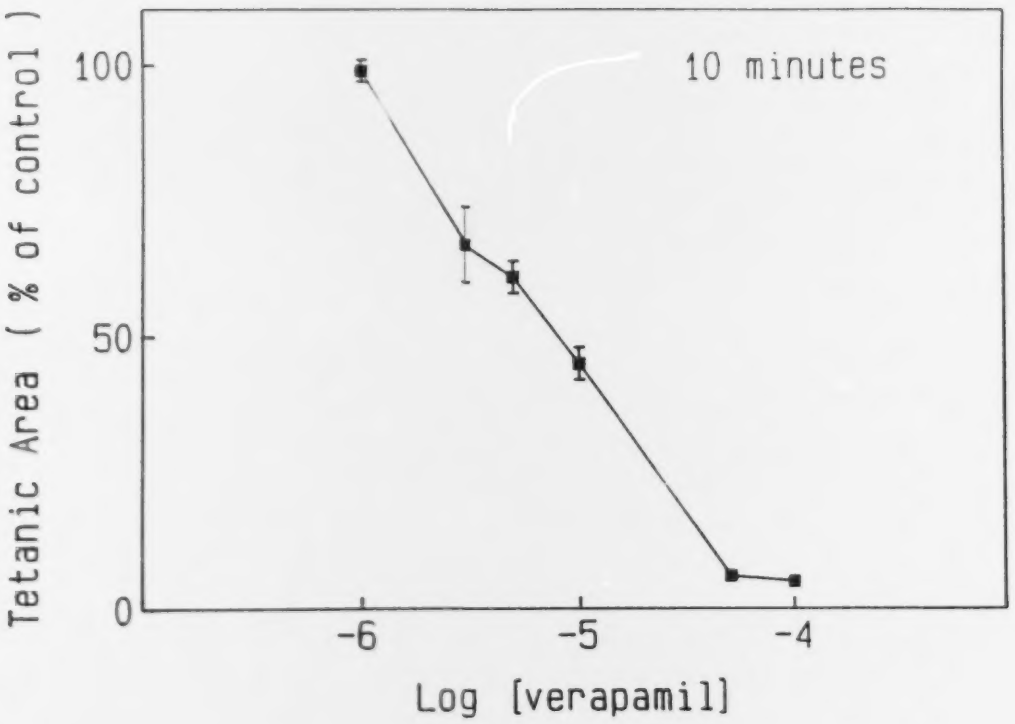


FIGURE 15: Effects of 10-min exposures to increasing concentrations of verapamil on areas of 2 sec tetani at 100 Hz. The numbers of experiments (n) at each concentration are given in the legend for Figure 14.

gradually during the 2 sec stimulus train (see Figure 12). In contrast, verapamil produced a variable depression pattern on the tetanic responses (Figure 16). Verapamil, at the concentration of 3 x 10^{-6} M (n = 5) like nitrendipine, produced only a gradual decline in tetanic tension beginning 0.5 to 1 sec after the start of the 2 sec stimulus train (Figure 16, A). At concentrations of 5 x 10^{-6} M (n = 3) to 10^{-5} M (n = 3) verapamil, there was often a rapid decline in tension to zero followed by another rise in tension during the stimulus train (Figure 16, B). This depressant effect of verapamil at a concentration of 5 x 10^{-6} M was reversed by using lower stimulation frequencies such as 50 Hz (Figure 17). Increasing the concentration of verapamil to 5 x 10^{-5} or to 10^{-4} M caused a rapid block of tetanic contraction during the stimulus train at 100 Hz for 2 sec (Figure 16, C). At these high concentrations of verapamil (10^{-4} M), in some muscles only, the maximum tetanic tension decreased to 5 - 10% of control values (n : 3), but twitch responses were not reduced. On the other hand, in some muscle fibres (n: 2) at the same concentration of verapamil, both the maximum tension of twitch and tetanic responses were reduced to 50% and 5 - 10% of control values respectively, but no seasonal effect was observed.

Except at these very high concentrations, verapamil did not affect the maximum tension developed in twitch and tetanic responses at 100 Hz. For example, in the experimental results plotted in Figure 18, exposing a frog toe muscle to verapamil at the concentration of 10^{-5} M, had essentially no effect on the development of the maximum twitch or tetanic tensions.

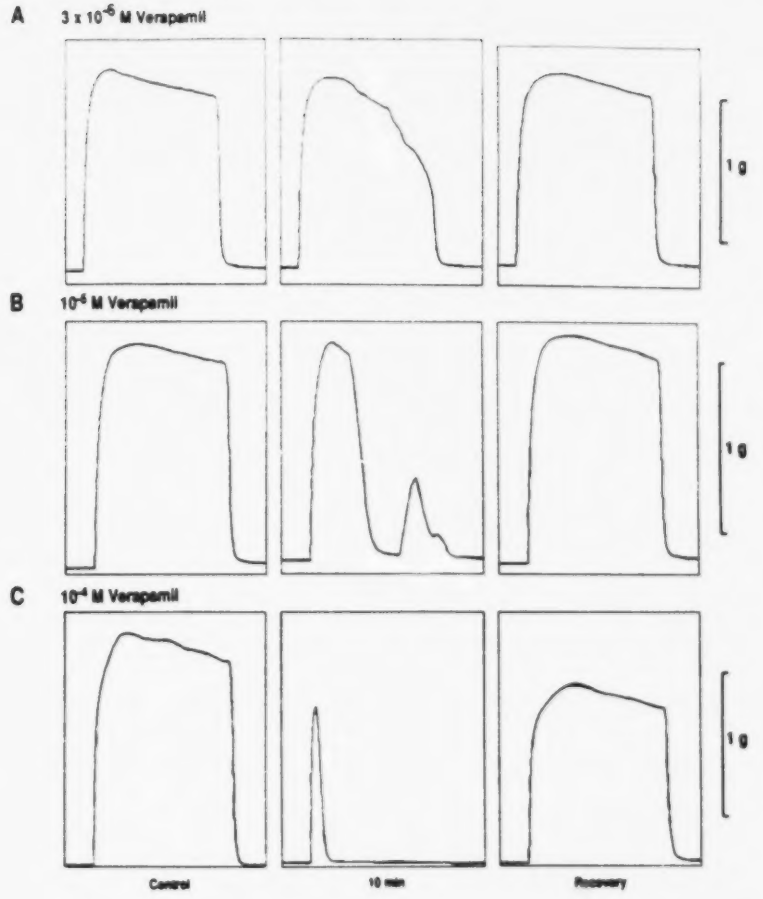


FIGURE 16: Effects of different verapamil concentrations on the tetanic contractions of frog skeletal muscle over 2 sec. The concentrations of verapamil used are indicated in the figure. Stimulation frequency = 100 Hz.

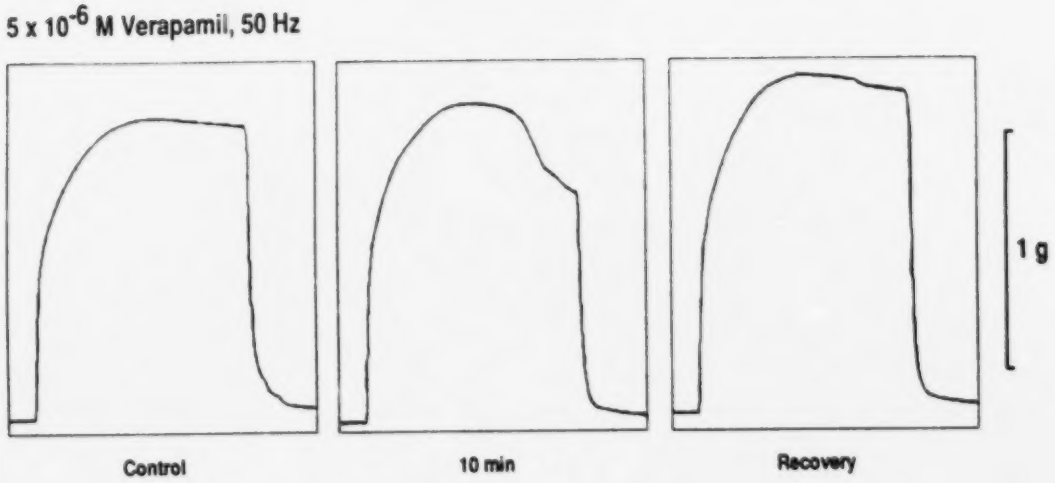


FIGURE 17: Tetanic response elicited by 50 Hz stimulation frequency for 2 sec in the presence of 5 x 10^{-6} M verapamil.

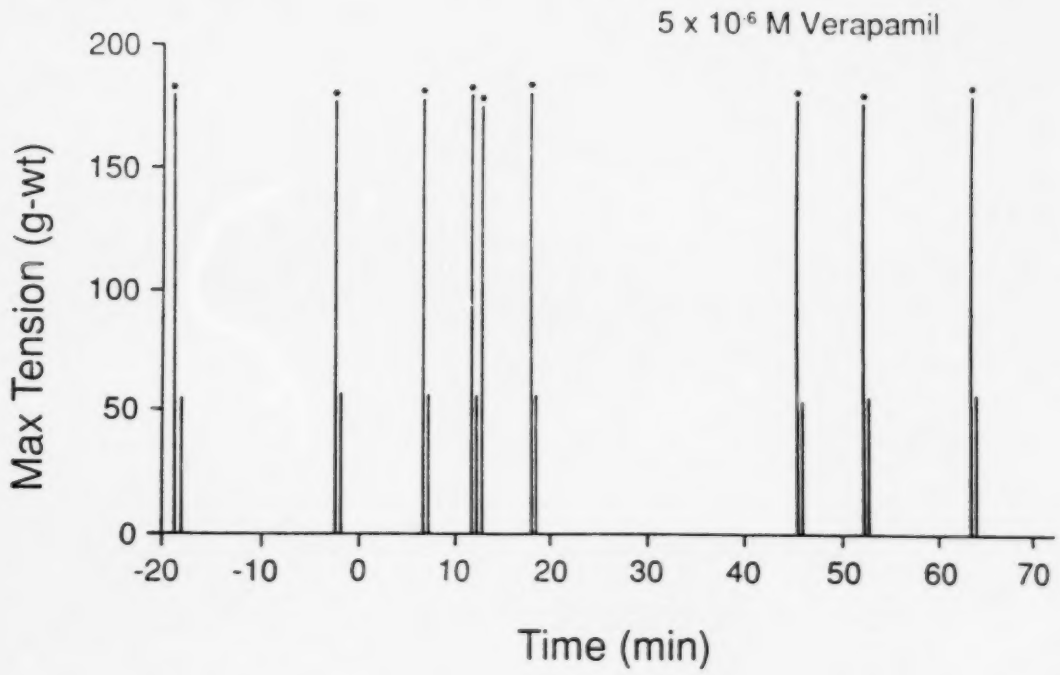


FIGURE 18: Chronic lack of an effect of 5 x 10^{-6} M verapamil on the maximum tensions of twitches and tetani evoked at 100 Hz for 2 sec. The muscle was placed in the drug containing solution at time 0. Tetani indicated by an * at the top of the line. All the responses obtained in a single experiment are shown.

TABLE III

Summary of Experimental Results with Different Concentrations of Verapamil

Concentr. of Verapamil (M)	(% of Control) Twitches / (% of Control) Tetani	5 min	10 min	15 min	30 min	45 min	60 min
10^{-4} n = 5	Twitches	99.60 ± 1	100.00 ± 0.45	98.20 ± 1.65	95.00 ± 2.17	93.80 ± 2.61	95.60 ± 1.94
	Tetani	100.80 ± 1.28	99.20 ± 2.17	100.60 ± 1.83	99.40 ± 1.99	99.00 ± 2.30	99.60 ± 1.63
3×10^{-4} n = 5	Twitches	101.00 ± 1.50	100.00 ± 2.02	100.00 ± 1.64	97.40 ± 1.91	98.00 ± 1.47	98.00 ± 1.50
	Tetani	70.00 ± 3.43	67.00 ± 6.6	62.00 ± 7.77	54.00 ± 4.27	53.00 ± 7.53	55.00 ± 7.27
5×10^{-4} n = 3	Twitches	96.33 ± 3.18	96.67 ± 3.34	94.33 ± 3.29	95.33 ± 1.84	98.00 ± 1.53	96.67 ± 2.19
	Tetani	60.00 ± 5.04	60.67 ± 3.34	54.67 ± 2.73	64.33 ± 11.36	53.00 ± 7.65	53.00 ± 8.36
10^{-3} n = 3	Twitches	102.00 ± 0.00	101.67 ± 0.41	102.00 ± 0.71	99.33 ± 0.82	100.67 ± 0.41	102.33 ± 1.48
	Tetani	44.33 ± 5.61	44.67 ± 3.29	41.00 ± 4.62	48.33 ± 6.85	58.33 ± 8.68	40.33 ± 4.18
5×10^{-3} n = 3	Twitches	106.67 ± 5.24	106.67 ± 4.91	107.67 ± 5.21	110.67 ± 5.93	110.33 ± 5.67	110.00 ± 5.67
	Tetani	6.33 ± 1.86	6.00 ± 1.16	5.33 ± 0.66	5.33 ± 0.34	5.33 ± 0.34	5.33 ± 0.34
10^{-2} n = 3	Twitches	106.00 ± 24.85	110.67 ± 23.89	112.33 ± 26.20	114.00 ± 28.71	113.33 ± 29.90	107.00 ± 28.04
	Tetani	7.00 ± 1.00	4.50 ± 0.50	5.00 ± 1.00	4.50 ± 0.50	4.00 ± 1.00	3.50 ± 1.50

4.2 Intracellular Recordings of Repetitive Action Potentials

4.2.1 Effects of Nitrendipine on Action Potentials in Toe Muscles

By using the intracellular recording method described in Section 3.2.2.1, it was possible to record the action potentials during repetitive stimulation at the rate used to produce the tetanic contractions (i.e. 100 Hz for 2 sec). Even so, the shorter the length of the stimulus train, the less likely it was to damage a muscle fibre. It was observed also that more reliable results were obtained when the Ringer's solution had been bubbled with a 99.5% O_2 - 0.5% CO_2 gas mixture for at least 10 min before placing the Ringer's in the muscle bath (see Method section, 3.2.2.1).

Records obtained in typical experiments of this type are shown in Figure 19. At the duration of 0.5 sec tetani (Fig. 19A), each action potential was fully recorded. However, with longer tetani (Fig. 19B and C), due to the limited time resolution of our digital oscilloscope, the peaks of the spikes were usually missed and an aliasing effect was seen. Even so, it becomes obvious on the recordings when there is a dropout of action potentials during a tetanus as often occurs when applying high verapamil concentrations (see Result section, 4.2.2) but does not occur when applying nitrendipine.

Nitrendipine at concentrations of 10^{-5} M (n: 3) and 10^{-4} M (n: 5) did not affect the amplitudes of repetitively occurring action potentials (see Figure 19.)

During the tetanic stimulation at 100 Hz the muscle fibre membrane depolarized relatively rapidly during the first 0.5 sec. Usually this was followed by a continuing, slowly developing, smaller depolarization with longer stimulus trains. After the stimulation train the muscle fibre remained depolarized and it took several seconds for it to repolarize. This long-lasting after-depolarization (LAP) is produced by the accumulation of K^+ ions in the T-tubules during the tetanus (see Introduction section, 1.3.2).

Eight complete experiments were conducted - three with 10^{-5} M and five with 10^{-4} M

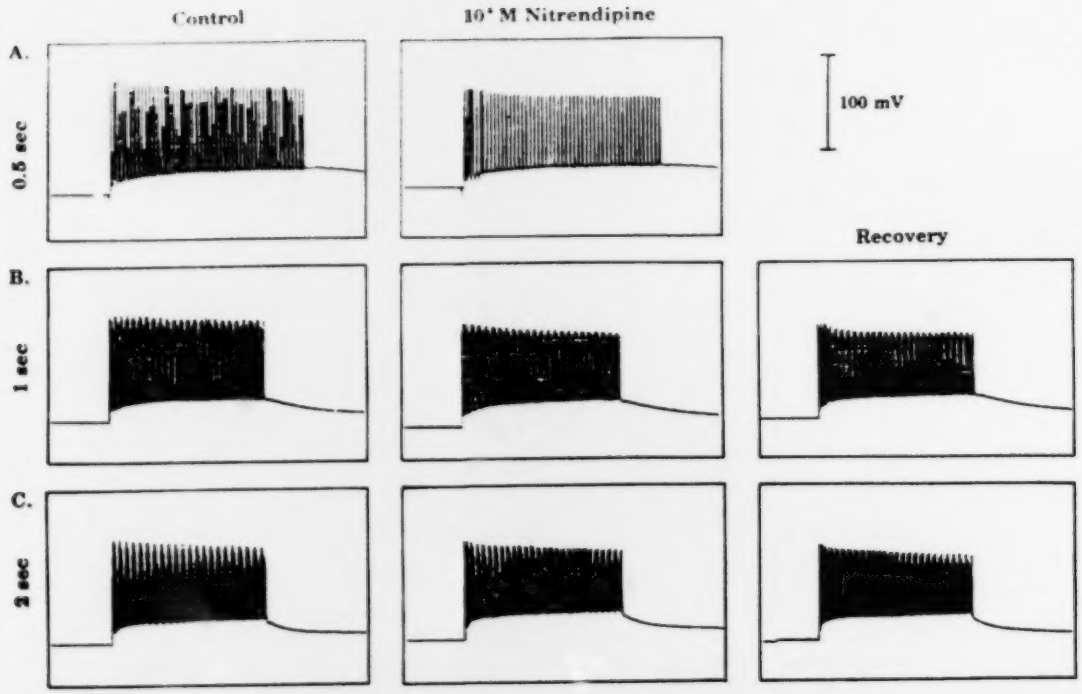


FIGURE 19: Intracellularly recorded potentials for tetanic simulation trains in frog toe muscle fibers. Recordings from different single fibres are presented in A, B and C. The lengths of the stimulus trains at 100 Hz are listed at the left of the recordings. The muscles were equilibrated in the drug solutions for 10 min or more before the recordings in the actual panels were made, and were placed back in Ringer's solution for at least 10 min before the recovery recordings were made.

TABLE IV

LAPs recorded in the presence and in the absence of nitrendipine

Times, length of exposure time to nitrendipine when the recordings were made.

Muscle	Nitrendipine Conc.	Tetanus Duration	Initial Maximum Amplitudes				
			Control		With nitrendipine		
			n	Mean LAP[a]	*n*	Mean LAP[a]	Times
	M	*sec*		*mV*		*mV*	*min*
8241	10^{-5}	0.5	1	25.3	1	21.7	10
		1.0	3	25.3	2	25.9	11, 21
		2.0	1	25.3	2	28.9	15, 19
8242	10^{-5}	0.5	1	25.3			
		1.0	3	22.5	2	29.5	11, 16
		2.0	3	25.9	1	31.3	14
		4.0	1	28.9			
8230	10^{-5}	0.5	3	20.9	1	16.9	9
		1.0	3	19.3	3	23.2	10, 13, 14
Means ± SE				23.5 ±0.98		25.3 ±2.19	
8161	10^{-4}	0.5	4	14.2	4	26.8	14, 24, 29, 44
		1.0	2	17.5	2	27.7	17, 26
		2.0			1	25.3	20
8162	10^{-4}	0.5	2	13.3	1	19.3	9
		2.0			1	18.0	10
8010	10^{-4}	0.5	2	22.9	2	25.5	10, 26
7300	10^{-4}	0.5	3	23.3	2	28.3	10, 12
7301	10^{-4}	0.5	6	20.7	2	22.9	11, 13
Means ± SE				18.7 ±1.76		25.0* ±1.39	

[a] When *n* is >1 the simple means are listed.

* Significantly different from controls at P = .05 (paired *t* test).

nitrendipine. Experimental results showing the effect of nitrendipine application on the maximum amplitudes of LAPs measured at the end of the repetitive stimulus trains, are tabulated in Table IV. The only effect of nitrendipine was to increase the initial amplitude of the LAP. This increase was not significant with 10^{-5} M nitrendipine but it averaged 6.3 mV in 10^{-4} M nitrendipine and was statistically significant.

4.2.2 Effects of Verapamil on Action Potentials in Toe Muscles

Although there was a significant depression of tetanic responses at the concentration of 3×10^{-6} M (see Figures 14 A and 15), at the same concentration there was no blockade of action potentials during 2 sec trains at 100 Hz of tetanic stimuli (n: 3, Figure 20). At the concentrations of 5×10^{-6} M and above, using 100 Hz stimulation frequency, verapamil always blocked the development of some action potentials during the stimulus train. Depending on the concentration used, the time to block the development of repetitive action potentials was about 1.5 sec to tens of milliseconds (Figure 20). Occasionally, we were also able to record the repetitive action potentials occurring in a biphasic manner (Figure 21).

This biphasic development of action potentials seen in the intracellular recordings were consistent with the biphasic force traces often seen in tension measurements (Figure 16B).

During intracellular recordings of repetitive action potentials, it was found that the blockade of Na^+ action potentials was in a use-dependent manner. The use-dependent blockade of Na^+ action potentials varied with the stimulus frequency and the concentration of verapamil used.

The use-dependent blockade of Na^+ action potentials was reversed by using lower stimulation frequencies such as 25 and 50 Hz. The effects of lowering the stimulation frequencies on the blockade of Na^+ action potentials are shown in Figure 22. Only the loss of few responses

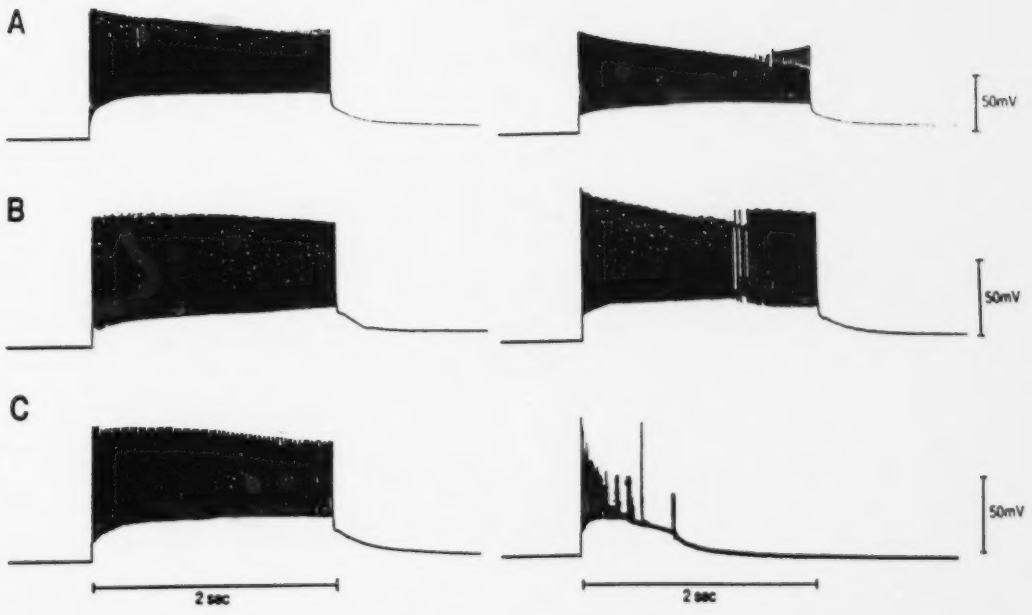


FIGURE 20: Effects of different verapamil concentrations on intracellularly recorded action potentials during 2 sec stimulus trains at 100 Hz. A, B, & C different toe muscles. On the left control recordings. On the right responses to the stimulus train recorded in different fibres after 10 min in solutions with verapamil; in A, 3×10^{-6} M; in B, 5×10^{-6} M; and in C, 10^{-4} M. Some small stimulus artifacts can be seen in between the action potentials. These have been removed from the end of the record in C when the action potentials were blocked.

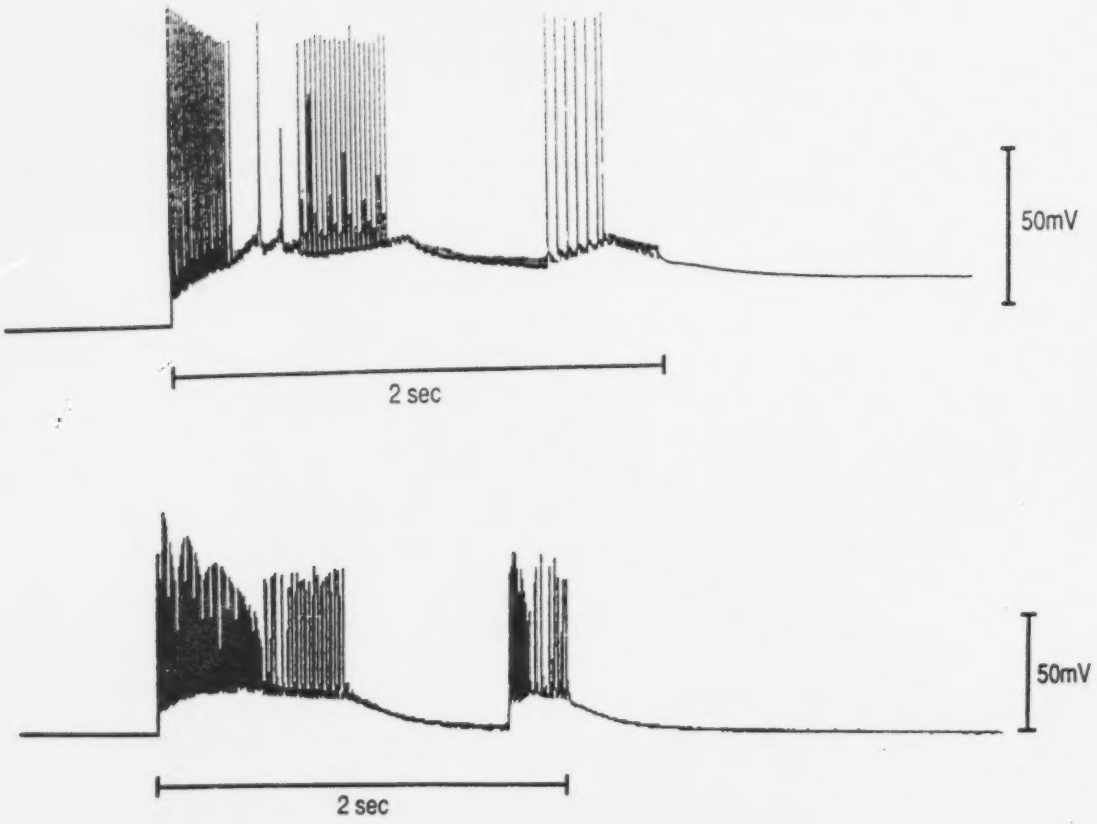


FIGURE 21: Blockade of action potentials and rebursting during a 2 sec stimulus train at 100 Hz recorded in a toe muscle fibre exposed to 5 x 10^{-6} M (at the top) and 10^{-5} M (at the bottom) verapamil for 10 min. Some stimulus artifacts can be seen between these action potentials during repetitive activities. Between the bursts these artifacts have been reduced in size. Due to the limited time resolution of the digital oscilloscope used in the lower recording, the peaks of the spikes are usually missed and aliasing effect is seen.

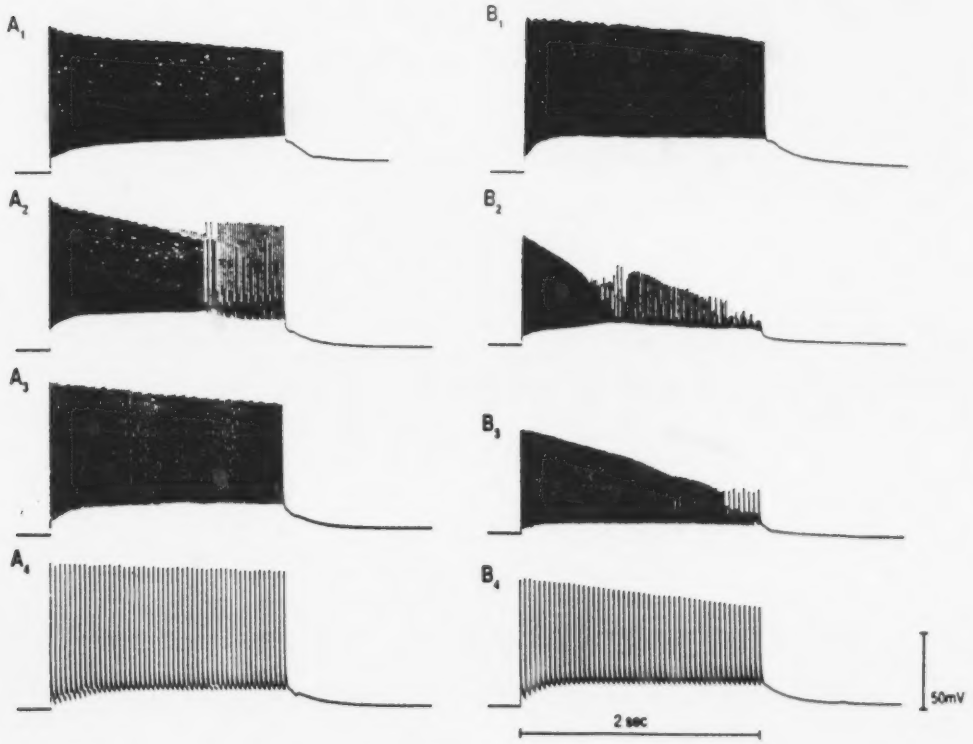


FIGURE 22: Intracellularly recorded action potentials for tetanic stimulation trains in frog toe muscle fibres. Recordings from different single fibres are presented in column A_1 through A_4 and B_1 through B_4. Control recordings are a_1 and B_1. Verapamil at the concentration of 5×10^{-6} M and 10^{-5} M used in recordings A_2 - A_4 and B_2 - B_4 respectively. Frequency of stimulations used during recordings are 100; 100; 50 and 25 for A_1, B_1; A_2, B_2; A_3, B_3; and A_4, B_4, respectively.

near the end of the 2 sec stimulus train occurred in the presence of 5 x 10^{-6} M verapamil (n = 3) and this was prevented by lowering the stimulus frequency to 50 Hz (Figure 22, A). A larger depression of repetitive action potentials was produced by 10^{-5} M verapamil (n = 3) and was only eliminated by reducing the stimulus frequency to 25 Hz (Figure 22, B).

4.3 $^{45}Ca^{2+}$ Efflux Studies in Rabbit T-Tubule Membrane Vesicles in the Range of LAPs During Tetanic Contractions

4.3.1 Introduction

Skeletal muscle T-tubules are the richest known source of L-type VSCCs (Fosset et al., 1983). Despite their highly concentrated presence in T-tubules, the functional role of these channels has not been unequivocally established. Isolated T-tubule membrane vesicles have been used to measure the functional responses of VSCCs (Dunn, 1989). In these experiments changes in potential across T-tubule membrane vesicles were induced by isosmotic exchange of choline chloride for potassium gluconate. This replacement of both cations and anions has been used previously to generate membrane potentials in SR and T-tubule vesicles (Ikemoto et al., 1984) and has been shown to reduce the swelling of vesicles that occurs with cation replacement alone. Neither choline (McKinley and Meissner, 1978) nor gluconate (Ikemoto et al., 1984) is appreciably permeable through T-tubule membrane vesicles. Under these conditions, if it is assumed that in the presence of valinomycin permeability of Cl^- << the permeability of K^+, then the membrane potential across the T-tubule vesicles can be predicted by the Nernst equilibrium potential for potassium ions (at 20 °C):

$$E_m - E_k - 58 \; Log \frac{[K^+]_o}{[K^+]_i}$$

In previous studies using the potential sensitive fluorescent dye, diS-C_3-(5), it has been shown that the potentials predicted by the Nernst equation, can be developed in T-tubule vesicles (see Dunn, 1989).

It has also been shown that the VSCCs in these T-tubule membrane preparations display the expected voltage dependence of activation and are modulated by calcium channel blockers and activators (Dunn, 1989; Bhat et al., 1992). Since the amplitude of LAPs in amphibian muscle fibres are in a similar range of LAPs in mammalian muscle fibres (Adrian and Bryant, 1974), in these experiments, we have investigated whether the VSCCs in isolated T-tubule membrane preparations can function to permit Ca^{2+} influx into the muscle fibres in the voltage range of LAPs.

4.3.2 $^{45}Ca^{2+}$ Flux Responses in the Range of LAPs

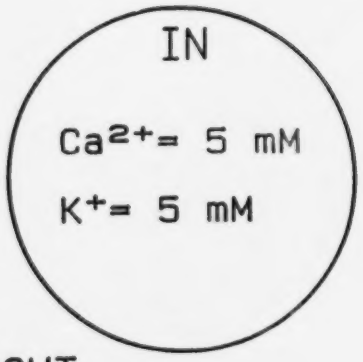
It was previously reported (Dunn, 1989) that if $^{45}Ca^{2+}$ loaded membrane vesicles prepared in low K^+ (5 mM) buffer are diluted into buffer containing 150 mM K^+ in the presence of valinomycin, the vesicles develop an inward positive membrane potential (estimated at an outward potential of -86 mV, mimicking the resting state of the cell in an inside out vesicle). Subsequent depolarization causes efflux of $^{45}Ca^{2+}$ from the vesicle, and this flux response is modulated by inorganic and organic calcium channel ligands. Similar experimental procedures were applied in the present study. Using this two-step protocol, the T-tubule vesicles were first repolarized to the estimated potential of -80 mV from 0 mV, and after a 5 min period in this repolarizing buffer (120 mM potassium gluconate, 30 mM choline chloride, 10 mM Hepes-Tris, pH:7.4), membranes were exposed to a partial depolarizing buffer (54 mM potassium gluconate, 96 mM

choline chloride, 10 mM Hepes-Tris, pH:7.4) for 20 sec to reach an estimated potential of -60 mV, which is similar to the potential that one would observe during LAPS in intact muscle fibres. Applying this two-step experimental procedure, it was found that during the 5 min repolarization period, there was no significant $^{45}Ca^{2+}$ efflux. It was found that 93% ± 6 (SEM, n: 22) of control $^{45}Ca^{2+}$ values were retained under these conditions. On the other hand, after first repolarizing the vesicles to an estimated -80 mV, subsequent depolarization of $^{45}Ca^{2+}$-loaded vesicles to an estimated -60 mV, resulted in a significant $^{45}Ca^{2+}$ flux response that presumably occurs through VSCCs on these T-tubule membrane vesicles. Schematic representation of this two step protocol is shown in Figure 23. Experimental results of these studies are presented in Figure 24. Only 31% ± 3 (SEM, n: 34) of control $^{45}Ca^{2+}$ remained after partial depolarizations of T-tubule vesicles.

4.3.3 Effects of Inorganic Calcium Channel Blockers on $^{45}Ca^{2+}$ Flux Responses

The effects of the inorganic calcium channel blockers $CoCl_2$ (1 mM), $NiCl_2$ (1 mM), and $LaCl_3$ (1 mM), on calcium flux responses described above, have been examined to characterize the pharmacological properties of these fluxes. Figure 25 shows the results of experiments carried out using the two-step protocol described previously, in the presence of the inorganic calcium channel blockers, Co^{2+}, Ni^{2+} and La^{3+}. Co^{2+}, Ni^{2+}, or La^{3+} did not affect the amount of $^{45}Ca^{2+}$ retained in the vesicles under control circumstances i.e. without a potential difference across the vesicle membranes. However, if the T-tubule membranes were first repolarized to an estimated potential of -80 mV and then depolarized to an estimated potential of -60 mV, there was a significant flux response in the 20 sec partial depolarization period. This flux response was partially inhibited by $CoCl_2$ (2 mM) and $NiCl_2$ (2 mM). Simultaneous application of Co^{2+} (1 mM) and Ni^{2+} (1 mM) resulted in a potentiated inhibitory response, thus increasing the extent

(A)

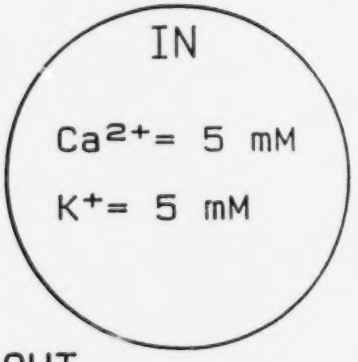


OUT

Ca^{2+}= 5 mM

K^+= 5 mM

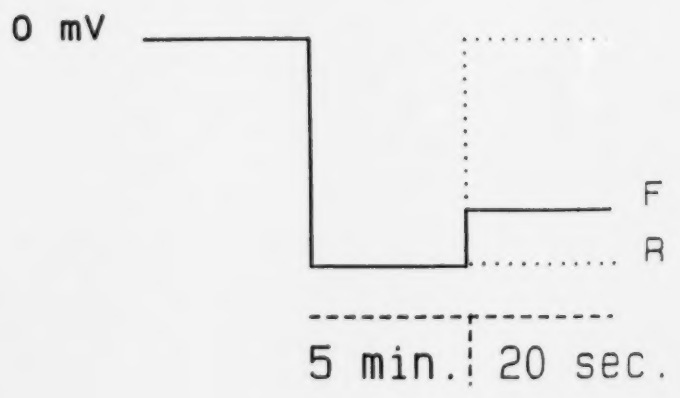
(B)



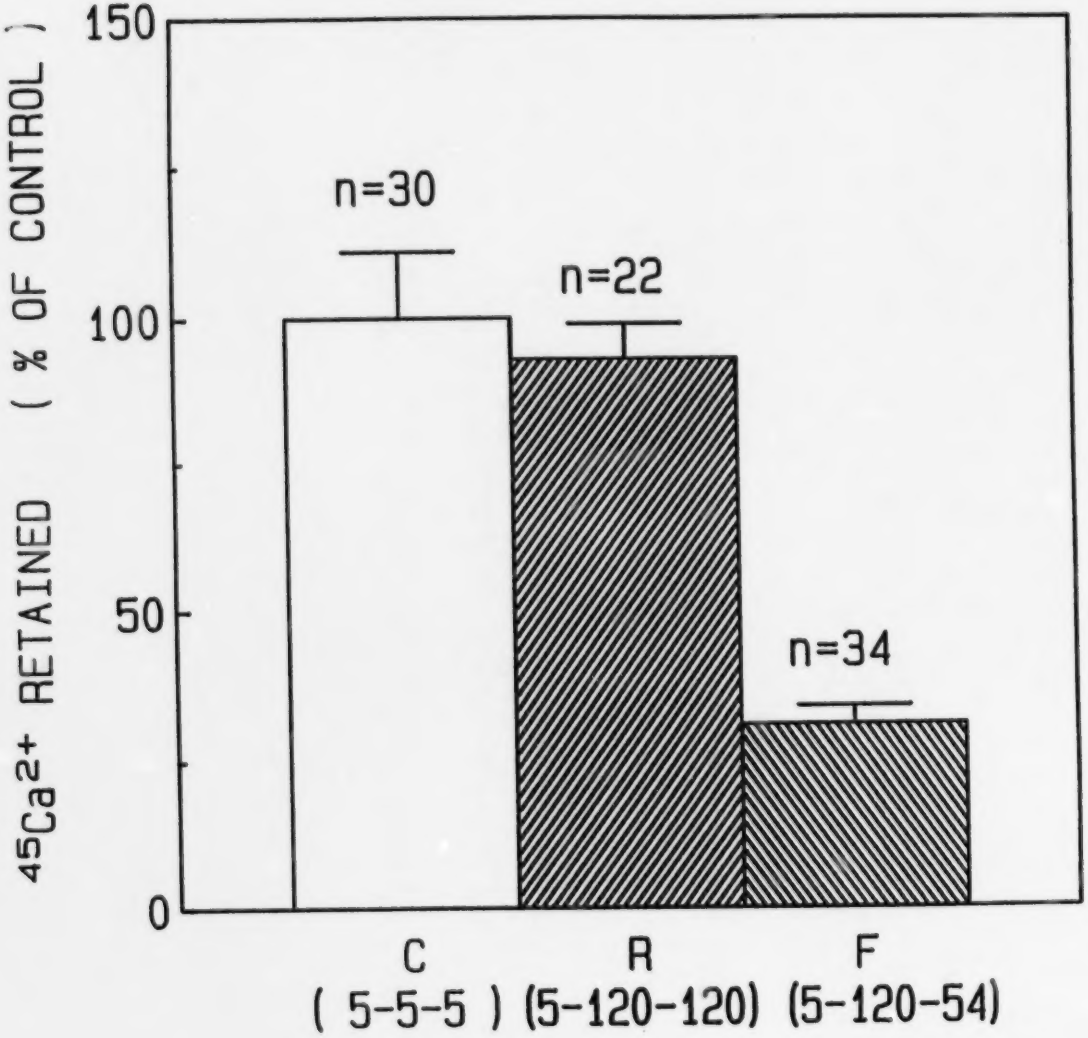
OUT

Ca^{2+}= 0.1 μM

K^+= 120 - 54 mM



96


150
100
50
0
n=30
n=22
n=34
$^{45}Ca^{2+}$ RETAINED (% OF CONTROL)
C
(5-5-5)
R
(5-120-120)
F
(5-120-54)

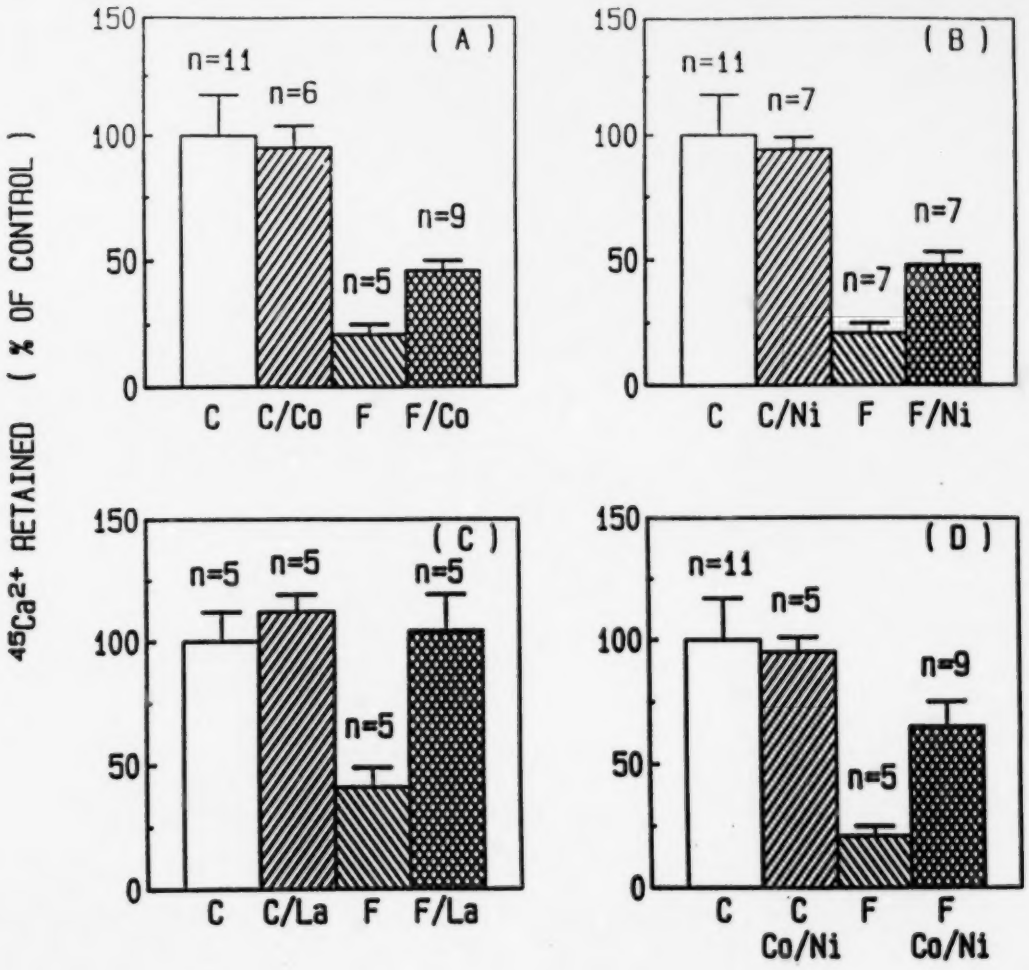


FIGURE 25: Effects of the inorganic calcium channel blockers, Co^{2+}, Ni^{2+}, and La^{3+} on calcium flux responses in T-tubule vesicles. In each panel, histograms marked C represent vesicles that underwent no change in membrane potential as in the control (C) experiments of Figure 24. Those marked (F) were first repolarized to an estimated -80 mV and then depolarized to -60 mV to initiate the flux response as in the experiments marked (F) in Figure 24. In each experiment, the effects of the channel blockers were tested under both control and flux conditions as indicated in the figures. The four panels show the effects of 2 mM $CoCl_2$ (A), 2 mM $NiCl_2$ (B), 1 mM $LaCl_3$ (C) and the combined presence of 1 mM $CoCl_2$ and 1 mM $NiCl_2$ (D).

TABLE V

Summary of the effects of inorganic calcium channel blockers on calcium flux responses of T-tubule vesicles

	Co^{2+} (2 mm)	Ni^{2+} (2 mm)	La^{3+} (1 mm)	Co^{2+} + Ni^{2+} (1 mm) (1 mm)
Control (5-5-5mM K^+)	100.00 ± 16.53 n = 15	100.00 ± 16.53 n = 15	100.00 ± 12.00 n = 5	100.00 ± 16.53 n = 15
Control + Inorg. Blocker (5-5-5mM K^+)	94.35 ± 8.08 n = 6	94.17 ± 4.80 n = 7	112.17 ± 6.45 n = 5	94.49 ± 5.45 n = 5
Flux (5-120-54mM K^+)	20.56 ± 3.50 n = 5	20.56 ± 3.50 n = 5	41.08 ± 8.00 n = 5	20.56 ± 3.50 n = 5
Flux + Inorg. Blocker (5-120-54mM K^+)	45.49 ± 3.55 n = 9	47.72 ± 4.82 n = 7	104.03 ± 14.53 n = 5	64.42 ± 9.63 n = 9

(arithmatic mean ± standard error mean of values indicating % of control values)

of inhibition. In the presence of $LaCl_3$ (1 mM), flux responses were completely inhibited. Experimental results obtained are tabulated in Table V.

4.3.4 Effects of Organic Calcium Channel Antagonists on $^{45}Ca^{2+}$ Flux Responses

It was previously reported that when T-tubule membranes were polarized to an estimated -80 mV and depolarized to 0 mV, organic calcium channel antagonists inhibited $^{45}Ca^{2+}$ efflux responses (Dunn, 1989; Bhat et al., 1992). In the present experiments, $^{45}Ca^{2+}$ flux occurring upon partial depolarization to potentials in the same voltage-range as LAPs in intact fibres, were significantly inhibited by most of the organic calcium antagonists used, including verapamil and nifedipine. (±)-Nitrendipine, on the other hand, had no effect on these $^{45}Ca^{2+}$ flux responses. A summary of these observations with organic calcium channel antagonists are shown in Figure 26. Our experimental results are also presented in Table VI.

4.3.5 Effects of Organic Calcium Channel Agonists, (±) Bay K8644 on $^{45}Ca^{2+}$ Flux Responses

Previous studies have reported that the DHP agonist Bay K8644 could stimulate $^{45}Ca^{2+}$ flux responses both under control conditions (no changes in membrane potential) or after depolarization from a repolarized state (Dunn, 1989; Bhat et al., 1992). These observations, made by depolarizing the T-tubule vesicles from -85 mV to 0 mV, are also seen under partially depolarizing conditions. The effect of the calcium channel agonist, (±) Bay K8644 (1 μM) on partial depolarization-induced $^{45}Ca^{2+}$ efflux in T-tubule vesicles is shown in Figure 27. Even in the control situation, where $^{45}Ca^{2+}$ loaded vesicles underwent no changes in membrane potential, 1 μM Bay K8644 caused a significant decrease in the amount of $^{45}Ca^{2+}$ retained in T-tubule vesicles. Efflux of $^{45}Ca^{2+}$, induced by partial depolarization of vesicles following an initial

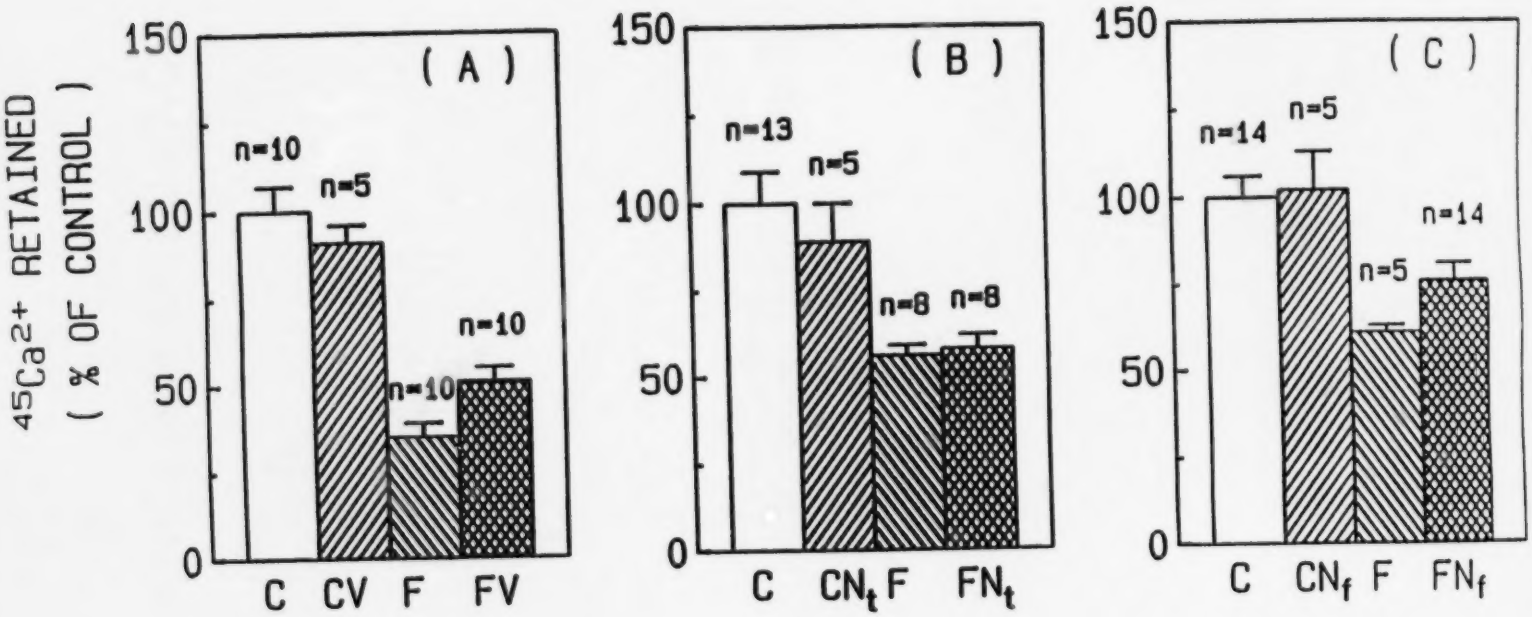


FIGURE 26: Effects of organic calcium channel blockers on calcium flux responses in T-tubule vesicles. Experiments were carried out under both control (C) and flux initiated by partial depolarization from -80 mV (F) as in Figure 24. The effects of 100 μM verapamil, 10 μM nitrendipine and 10 μM nifedipine under both control and flux conditions are illustrated in panels (A), (B) and (C) respectively.

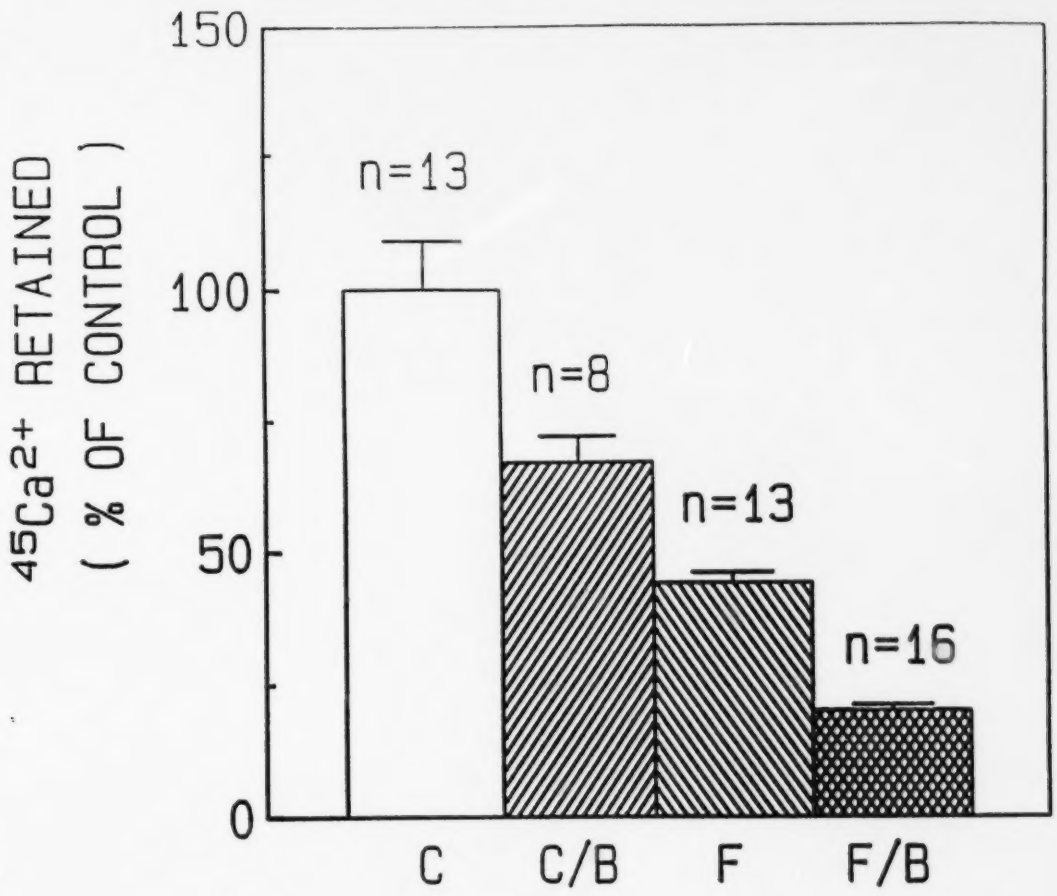


FIGURE 27: Stimulation of $^{45}Ca^{2+}$ efflux by (±) Bay K8644. Experiments were carried out as described in the legend to Figure 24, except that in C/B and F/B 1 μM (±) Bay K8644 was included in the control and flux induced by partial depolarization to -60 mV, respectively.

repolarization phase, is also stimulated by Bay K8644 (1 μM). A summary of the results of experiments with (±) Bay K8644 is shown in Table VI.

4.3.6 Effects of (+) and (-) Enantiomers of (±) 202-791 on $^{45}Ca^{2+}$ Flux Responses

In a number of studies it has been shown that the effects of DHPs are stereospecific, (Franckowiak et al., 1985; Hof et al., 1985). The enantiomers of SDZ 202-791, for example, appear to have opposite effects with the (+) or (S) isomer acting as a channel activator and the (-) or (R) isomer being a channel blocker (Williams et al., 1985; Kokubun et al., 1986). Similar results have been obtained for the action of these ligands under the partial depolarization conditions used in the present study. Our experimental data are presented in Table IV. The results in Figure 28 demonstrate that whereas (-)-SDZ 202-791 (10 μM) significantly inhibited the flux response, (+)-SDZ 202-791 (10 μM) acted as an agonist.

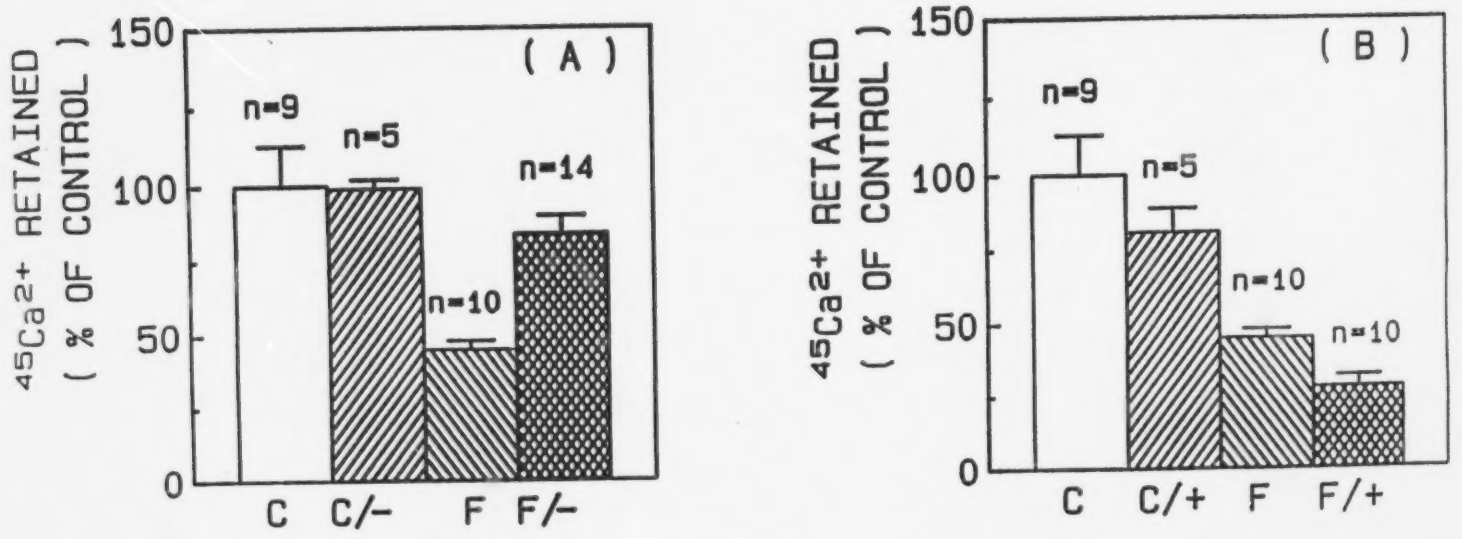


FIGURE 28: Effects of (-)-SDZ 202-791 and (+)-SDZ 202-791 on $^{45}Ca^{2+}$ flux responses induced by partial depolarization of T-tubule vesicles. The conditions used to measure control (C) and flux (F) responses were identical to those described in the legend to Figure 24. The data in (A) show the inhibition of flux by the antagonist (-)-SDZ 202-791 (10 μM) and (B) shows the potentiation of the flux response by (+)-SDZ 202-791 (10 μM).

TABLE VI

Summary of the effects of organic calcium channel ligands
on calcium flux responses of T-tubule vesicles

	(±)Bay K8644 (1 μM)	Nifedipine (10 μM)	Nitrendipine (10 μM)	Verapamil (100 μM)	(+) SZD 202 791 (10 μM)	(-) SZD 202 791 (10 μM)
Control (5-5-5mM K^+)	100.00 ± 8.57 n = 13	100.00 ± 6.28 n = 14	100.00 ± 9.20 n = 13	100.00 ± 7.48 n = 10	100.00 ± 13.00 n = 9	100.00 ±13.00 n = 9
Control + Inorg. Blocker (5-5-5mM K^+)	66.92 ± 5.43 n = 8	101.81 ± 10.75 n = 5	88.91 ± 10.63 n = 5	90.49 ± 4.51 n = 5	80.60 ± 8.00 n = 5	99.4 ± 3.12 n = 5
Flux (5-120-54 mM K^+)	44.18 ± 2.00 n = 13	61.40 ± 1.86 n = 5	56.36 ± 2.77 n = 8	35.25 ± 3.75 n = 10	45.00 ± 2.94 n = 10	45.00 ± 2.94 n = 10
Flux + Inorg. Blocker (5-120-54 mM K^+)	19.46 ± 1.0 n = 16	75.27 ± 4.79 n = 14	58.00 ± 3.68 n = 8	51.05 ± 3.47 n = 10	28.20 ± 3.80 n = 10	83.14 ± 5.18 n = 14

(arithmatic mean ± standard error mean of values indicating % of control values)

5. DISCUSSION

Everything should be made as simple as possible but not simpler.

- A. Einstein

5.1 Potentiation of Twitch Responses in Ca^{2+}-Free Solutions

The effect of Ca^{2+} removal on twitch responses has been the subject of numerous studies (Caputo and Gimenez, 1967; Sandow et al., 1975; Barret and Barret, 1980; Frank, 1982a,b). After exposure of muscle fibre to Ca^{2+}-free solutions, an initial potentiation of twitches has been a common finding in these studies. During our experiments, there was also an initial twitch potentiation in Ca^{2+}-free solutions containing EDTA. In the concentration range of 10^{-5} to 2.5 x 10^{-4} M EDTA, this initial potentiation phase lasted for 5 - 15 mins. With increasing concentrations of EDTA (up to 5 x 10^{-3} M), after a 5 - 10 min period, the amplitude of twitches began to decrease (see Figure 8). Very similar results regarding the time course of twitch responses in Ca^{2+}-free solutions have been reported in other studies (Oota et al., 1976; Frank, 1978a,b). It has been shown also that the large depolarizations in calcium-free solutions reported by some (Edman and Grieve, 1963; Milligan, 1965) but not all (Zacharova and Zachar, 1967, Gainer, 1968; Frank and Inoue, 1973) workers, are artifacts caused by inserting microelectrodes into muscle fibre membranes made more susceptible to damage by calcium-free solutions (Frank and Inoue, 1973). In any event, large depolarizations only appear after 10 min or more in calcium-free solutions (Frank and Inoue, 1973). Interestingly, the time period for the loss of twitch amplitudes is longer than required for free diffusion of Ca^{2+} out of the T-tubules of the muscle fibres but much quicker than required for an effective reduction in intracellular Ca^{2+} stores which takes several hours in Ca^{2+}-free solutions (Gilbert and Fenn, 1957; Frank, 1962; 1982a). This was also supported strongly by the lack of a relation between the size (length) of the T-tubular network and the time required to eliminate the twitches (Frank, 1982a). These pioneering experiments clearly showed the presence of extracellular binding sites for Ca^{2+} on the luminal surface of the T-tubular membrane and that the occupation of these sites by Ca^{2+} is required for twitch development (Bianchi, 1969; Frank, 1982a,b). The presence of extracellular

binding sites in T-tubules that are required for the release of Ca^{2+} has also been reported and well established in later studies (Luttgau, 1986; Brum et al., 1988; Pizzaro et al., 1988).

Different interpretations for the role of T-tubular membrane bound Ca^{2+} have been made in different studies (Frank, 1980; Rios and Pizzaro, 1991). According to the trigger Ca^{2+} hypothesis, occupation of extracellular binding sites for Ca^{2+} increases the binding (or affinity) of trigger Ca^{2+} located on the inner surface of T-tubules to this membrane. When the luminal Ca^{2+} concentration is lowered and fewer of these sites are occupied by extracellular Ca^{2+}, each action potential releases more trigger Ca^{2+} into the triadic junction and the twitch is potentiated (Frank, 1980; Bianchi, 1968, 1969). This is followed by a longer time interval (usually 1 - 10 min) during which the amount of trigger Ca^{2+} bound to tubular membranes is reduced to a level that cannot induce release of Ca^{2+} from SR (Oota et al., 1976; Frank, 1980).

When added to a Ca^{2+}-free solution in the appropriate concentrations, many multivalent cations can either prevent the loss of or restore K^+-induced contractures (Frank, 1962; Anderson and Edman, 1974) or twitches (Frank and Treffers, 1978; Frank and Rohani, 1982) previously eliminated by exposure to the zero Ca^{2+} solution. According to the trigger Ca^{2+} hypothesis, this is done by occupying the superficial binding sites thereby preventing the loss of trigger Ca^{2+} ions into the Ca^{2+}-free solution in the T-tubule lumina. The results obtained in other studies of the effects of extracellular Ca^{2+} removal on the twitch in isolated skeletal muscle fibre preparations also support the trigger Ca^{2+} hypothesis for E-C coupling (Oota et al., 1976; Frank, 1978a, 1978b, 1982).

5.2 Depression of Tetanic Contractions in Ca^{2+}-Free Solution

In some of the early studies, it was demonstrated that there is a small amount of extracellular Ca^{2+} entrance during twitch responses in frog twitch muscle fibres (Bianchi and Shanes, 1959; Curtis, 1966). During these studies, it was found that in whole muscle preparation an extra influx of radioactive $^{45}Ca^{2+}$ occurred during both a single twitch and K^+-induced contractures (Bianchi and Shanes, 1959; Weiss and Bianchi, 1965); in the presence of 1 mM external Ca^{2+}, the influx was estimated to be at 0.2 pM/cm^2 during a twitch. Bianchi and Shanes (1959) also observed that potentiating the fibre with nitrate led to some increase in the calcium influx and in the area of the twitch, a result that suggested a direct relation between twitch and calcium influx. Slightly higher values of calcium influx (0.73 pM/cm^2) in 1 mM external calcium, were then obtained in single muscle fibres by Curtis (1966). Furthermore, Bianchi and Narayan (1982a,b) also showed that during 1 Hz stimulation, a net uptake of Ca^{2+} occurs that is temporarily associated with progressively potentiating twitch amplitudes. Later, using an electron probe x-ray microanalysis technique, Somlyo et al. (1985) also found a significantly higher level of Ca^{2+} located in the terminal cisternae of SR after a 1.2 sec lasting tetanic contraction. Recently, Stefani and his colleagues using voltage clamp experiments conducted in frog skeletal muscle fibres suggested that the increased activation rate of Ca^{2+} currents during repetitive stimulations would cause increased flux of Ca^{2+} into the myoplasm during periods of intense activity such as tetanic contractions (Garcia et al., 1990a). Similar effects of repetitive stimulations on Ca^{2+} currents have also been reported in the isolated guinea pig ventricular myocytes (Mitra and Morad, 1986; Lee, 1987) and in frog ventricular myocytes (Schouten and Morad, 1989). In frog skeletal muscle fibres, the increase of the activation rate was independent of Ca^{2+} entry and was voltage-dependent, indicating that not entering Ca^{2+} but repetitive stimulations were required to produce such an effect (Garcia et al., 1990a). Furthermore, the

contribution of extracellular Ca^{2+} influx to intracellular Ca^{2+} transients measured by aequorin and arsenazo III has also been demonstrated in various studies (Blinks et al., 1978; Miledi et al., 1984). In frog twitch fibres, the aequorin transients during tetanic contractions (100 Hz stimulation rate for 2 sec) were distinctly more subject to fade in the Ca^{2+}-free solution (Blinks et al., 1978).

In our studies, it was found that tetanic contractions lasting 2 sec at 100 Hz stimulation rate were greatly depressed in Ca^{2+}-free solutions. Depending on the concentration of EDTA used (10^{-5} to 5×10^{-3} M), the area under the tension x time curve was decreased to 40 - 5% of the control values (See Fig. 8) during a 10 min recording period. On the other hand, unless EDTA was used at concentrations higher than 2.5×10^{-4} M (see Fig. 8) the maximum amplitude of single twitch responses were only potentiated during this time period. Recently, almost identical results have been reported by Stefani and his colleagues (Gamboa-Aldeco et al., 1989). In frog twitch muscle fibres, they have shown that the withdrawal of external Ca^{2+} did not affect twitch or the rising phase of tetanus, but greatly reduced the area of the tension-time curve of tetanic contracture. Kotsias et al. (1986), in mammalian skeletal muscle fibres also found a significant depression of fused tetanic responses in Ca^{2+}-free solutions.

In some of the experiments studying the effect of reduced extracellular Ca^{2+} on tetanic contractions, only a slight reduction of tension development has been reported (Luttgau and Spiecker, 1979; Dulhunty and Gage, 1988). Interestingly, both studies have measured the maximum tension development during tetanic contractions in Ca^{2+}-free solutions. We have also found that maximum tension development is relatively resistant to treatments with Ca^{2+}-free solutions (See Fig. 9), and similar results have also been observed in different studies (Kotsias et al., 1986; Gamboa-Aldeco et al., 1989). It appears that the depression of tetanic contractions in Ca^{2+}-free solutions starts only 200-300 ms after the beginning of repetitive stimulations (see

Fig. 7). In other words, the decrease of the area under the tension x time curve starts after tetanic tension reached its maximum amplitude. For this reason, in our studies (and also in Gamboe-Aldeco et al., 1989) the effects on tetanic contractions were measured by obtaining the time x tension area of each tetanic response.

Our experimental results, along with other studies, suggest that the influx of extracellular Ca^{2+} during a late phase of tetanic contractions plays an important role to maintain tension development.

Recently, in an interesting study, it was also found that during low frequency (1 Hz) repetitive stimulation of frog skeletal muscle fibre, reducing the influx of extracellular [Ca^{2+}] into the muscle fibres had some inhibitory role in the long term processes of skeletal muscle e-c coupling, such as development of fatigue (Williams, 1990; Williams and Ward, 1991; see also Williams and Barnes, 1989).

5.3 Potentiation of Twitch Responses by Nitrendipine and Verapamil

During our studies on the functional role of VSCCs in frog skeletal muscle fibres, we have employed the organic Ca^{2+} channel blockers, nitrendipine and verapamil as pharmacological tools to approach this problem. Both nitrendipine and verapamil were previously employed in our laboratory to investigate the effect of these drugs on e-c coupling process in skeletal muscle fibres (Frank, 1984; 1986; 1988). During our present studies, the effect of these drugs on twitches and tetanic contractions were comparatively studied to observe whether they have a differential effect on these two types of contractions. The results of this study were that both nitrendipine and verapamil in the concentration range of 10^{-6} to 10^{-4} M increased the maximum amplitude of twitch tension significantly. Meanwhile, the maximum amplitudes of tetanic contractions remained unchanged or were slightly decreased.

The twitch potentiating effect of organic Ca^{2+} channel blockers that we reported in our studies is quite consistent with most of the literature in this area. In vertebrate skeletal muscle including mammalian muscle, DHP-class Ca^{2+} channel antagonists such as nifedipine and nicardipine have been reported to have similar twitch potentiating effects (Griffiths and Taylor, 1982; Dulhunty and Gage, 1988; Singh and Dryden, 1988; Miller et al., 1989; Kawata and Hatae, 1990). The extent of the twitch potentiating effect (5 to 25% depending on the concentration of nitrendipine used) and the time of onset of the effect (5 to 10 min after drug application) are consistent with other studies. Another organic Ca^{2+} channel blocker, verapamil, a phenylalkylamine, also had a twitch potentiating effect although to a lesser extent (10% to 15%) during these studies on frog skeletal muscle fibres. Previous studies using verapamil and its methoxy-derivative D-600 have also reported the twitch potentiating effect of these drugs on frog twitch muscle fibres (Marwaha and Treffers, 1980; Helland et al., 1988; Kawata and Hatae, 1990). The onset (5 to 10 min) and the extent of the twitch potentiation found during our studies are also in agreement with other studies.

Although the site of the action of these organic Ca^{2+} channel blockers that results in their twitch potentiating effect has recently been studied extensively (Ohkusa et al., 1991; Roed, 1991; Viires et al., 1991), the exact nature of this effect remains unknown. It has been shown in many studies that the resting membrane potential of muscle fibres do not change even at a high concentration of these drugs (Marwaha and Treffers, 1980; Griffiths and Taylor, 1982; Hatae, 1986). Neither the rate of rise of the action potential nor its overshoot were significantly altered by the DHP-class Ca^{2+} antagonists (Griffiths and Taylor, 1982; Kawata and Hatae, 1990). Although the decrease of the maximum rate of rise and overshoot of action potential by verapamil and D-600 have been reported in a number of studies on frog twitch muscle fibres (Van der Kloot and Kita, 1975; Bondi, 1978; Frank, 1984), during twitch responses this effect on Na^+ channels

became apparent only at the concentrations higher than used in our studies (10^{-4} - 10^{-6} M of verapamil). But during repetitive stimulations, blockade of action potentials can be observed at lower concentrations of verapamil, due to the use-dependent blockade of Na^+ channels (Frank and Oz, 1991; Oz and Frank, 1992).

Thus, the twitch potentiating effect of organic Ca^{2+} channel blockers, (nitrendipine and verapamil) used in our studies does not seem to be due to their effect on action potential properties. On the other hand, an increased concentration of intracellular Ca^{2+} available for contractile machinery after the application of D-600 (Helland et al., 1988) has been shown in experiments using the Ca^{2+} sensitive photoprotein aequorin. Although the exact source of this increased $[Ca^{2+}]_i$ is not known, the twitch-potentiative action of these drugs have been attributed to lowering of mechanical threshold, activation of Ca^{2+} sequestration to SR, and/or induction of Ca^{2+} release from the SR (Sarmiento et al., 1984; Walsh, 1984; Singh and Dryden, 1988; Miller et al., 1989). Decrease of mechanical threshold by nifedipine has been reported in frog twitch muscle fibres (Neuhaus, 1987), but not in mammalian muscle fibres (Miller et al., 1989). On the other hand, in both mammalian and vertebrate skeletal muscle fibres, the enhancement of caffeine induced tension transients by organic Ca^{2+} channel antagonists has been a common finding in different studies (Bondi, 1978; Dryden and Singh, 1988 and Su, 1988). Since the caffeine induced contractures employ intracellular Ca^{2+} located in SR, both the activation of Ca^{2+} sequestration by an increased activity of Ca^{2+}-ATPase located mainly in longitudinal cisternae, and increased Ca^{2+} release from the SR would result in enhancement of caffeine contractures. Nitrendipine and nimodipine have been shown to increase Ca^{2+} sequestration and stimulate Ca^{2+}-ATPase activity to nearly the same extent as Ca^{2+} sequestration in SR vesicles of skeletal muscle, but nifedipine and verapamil were lacking such stimulant effect (Colvin et al., 1982; Sarmiento et al., 1984). Verapamil and diltiazem had an inhibitory effect on Ca^{2+}-ATPase of SR vesicles

(Wang et al., 1984) and decreased Ca^{2+} uptake by SR in skinned fibres (Su, 1988). In addition, the binding of Ca^{2+} channel blockers to the inner surface of the plasmalemma which results in a stereospecific inhibition of the plasmalemma-located Ca^{2+}-ATPase has also been demonstrated (Mas-Oliva and Nayler, 1980; Nayler et al., 1980). Hence, the effect of these drugs on the Ca^{2+} sequestration mechanism of SR is not unequivocally established yet. As another possible mechanism, increased Ca^{2+} release has also been reported in skinned fibre preparations (Su, 1988), in isolated SR vesicles (Wang et al., 1984) and in isolated intact skeletal muscle preparation (Gonzelez-Serratos and Phillips, 1984, Singh and Dryden, 1988). Although the Ca^{2+} release-increasing effects of organic Ca^{2+} channel antagonists appears to be a possible candidate for this effect, this has not been tested in a preparation (such as reconstitution of Ca^{2+} release channels into bilayers) that lacks a Ca^{2+}-ATPase. Furthermore, biochemical experiments show that these Ca^{2+} channel ligands have many interactions with intracellular proteins other than DHP binding sites located in T-tubules. Interaction of Ca^{2+} channel blockers with calmodulin has been reported in many studies (Boström et al., 1981; Johnson, 1983 and Lugnier et al., 1984). Binding sites with micromolar affinities for DHP-class Ca^{2+} channel ligands have also been identified in mitochondrial preparations (Zernig and Glossmann, 1988) which may be associated with peripheral benzodiazepine receptors (Cantor et al., 1984) or Na^{+} - Ca^{2+} exchangers (Vaghy et al., 1982). Other low affinity DHP binding proteins include the nucleoside transporter (Striessnig et al., 1985), Na^{+}-K^{+}-ATPase (Pan and Janis, 1984), calmodulin dependent cyclic AMP phosphodiesterase (Schaechtele et al., 1987) and the multidrug resistance related P-glycoprotein (Cornwell et al., 1987).

In view of these studies demonstrating a multiplicity of binding sites, it is possible that the observed twitch potentiating effects of Ca^{2+} channel ligands is due to interaction with their intracellularly located binding sites but the identification of these binding sites remains to be

established. Another possible mechanism for the twitch potentiating action of organic calcium channel ligands is that the low affinity binding sites for DHP-class channel ligands could also mediate this effect. It was shown that in the micromolar concentration range, not only DHP-class calcium channel antagonists, but also agonists of the DHPs induce a twitch potentiation (Viires et al., 1991 and our unpublished observations). Recently low affinity binding sites on, or related to, the high affinity DHP binding proteins have been found in skeletal muscle fibre (Dunn and Bladen, 1991; 1992).

In some of the recent studies, the twitch potentiating effect of Ca^{2+} channel blockers has been attributed to their binding to DHP receptors, rather than their effect on intracellular Ca^{2+} stores (Kawata and Hatae, 1990; Roed, 1991; Viires et al., 1991). An appropriate way to approach this problem would be the use of lipophilic and hydrophillic DHPs to conduct comparative studies.

5.4 Depression of Tetanic Responses by Nitrendipine

In order to investigate the functional role of VSCCs during tetanic contractions, we have used two chemically different types of organic Ca^{2+} channel blockers, nitrendipine and verapamil. During our experiments, nitrendipine at the concentration range of 10^{-6} to 10^{-4} M greatly depressed the area under the tetanic tension x time curve. Similar results using nifedipine and verapamil at similar concentrations to those used in our experiments have also been reported in various studies on frog and mammalian muscle fibres (Kotsias, 1986; Gamboa-Aldeco et al., 1989; Miller et al., 1989). In frog skeletal muscle fibres (Gamboa-Aldeco et al., 1989) and mammalian muscle fibres (Miller et al., 1989), nifedipine has been shown to decrease the area under the tetanic tension x time curve. On the other hand, in some of the earlier studies using mammalian muscle (Gallanth and Goettl, 1985; Dulhunty and Gage, 1988), DHP-class calcium

channel antagonists, nitrendipine and nifedipine did not show a depressant effect on tetanic responses. It is possible that nitrendipine or nifedipine does not dissolve as well using polyethylene glycol 400 or alcohol as in DMSO, or that powder preparation of these DHP-class antagonists had a decreased potency (see Frank, 1990). In our experiments, 10^{-5} M nitrendipine, for example, did not decrease the maximum tetanus tensions, and the decrease from peak tension after 300 ms, which was used to calculate "fade index" (Gallanth and Goettl, 1985), was very small. In addition, the recording of short lasting tetanic contractions, such as 0.8 sec, on long time scales, such as 10 sec to 3 min would also cause to be missed the effects we observed in frog skeletal muscle fibres. Furthermore, depending on the tissue, species and different DHP-class antagonist, varying results on calcium current measurements have been reported in numerous studies (Schwartz et al., 1985; Walsh et al., 1986). For example, in electrophysiological studies, Schwartz et al. (1985) have reported that nitrendipine (IC_{50} = 0.7 μM) was capable of blocking calcium currents in frog skeletal muscle fibres. On the other hand, in mammalian skeletal muscle fibres, even at relatively high concentrations (10 μM), nitrendipine had no blocking action on calcium current (Walsh et al., 1986). Furthermore, Coronado and Affolter (1985) have studied the electrophysiological properties of calcium channels from mammalian T-tubular preparation incorporated into lipid bilayer membranes. These channels had kinetic properties consistent with the skeletal muscle calcium currents and interestingly were sensitive to nitrendipine.

In our experimental conditions, nitrendipine at the concentration range of 10^{-6} to 10^{-4} M was used to probe the functional role of slow calcium channels in frog skeletal muscle. In earlier electrophysiological studies in frog skeletal muscle fibres, nitrendipine has been reported to block calcium currents in these concentration ranges (Schwartz et al., 1985).

During our electrophysiological studies, repetitively developed action potentials and LAPs

were intracellularly recorded in sodium Ringer's solution. In similar physiological conditions to those in which tetanic tension was recorded, we have shown that repetitive action potentials remained unchanged and LAPs increased slightly at the time that the area under tetanic tension x time was greatly depressed in the presence of nitrendipine. Even at the highest concentration of nitrendipine (10^{-4} M), no effect on repetitive action potentials was observed. In agreement with our experimental results with nitrendipine, in other studies it was also shown that DHP-class calcium channel antagonists do not change the action potential properties in frog (Kawata and Hatae, 1990) or in mammalian muscle fibres.

Furthermore, the release of Ca^{2+} from SR has been reported to be unaffected by DHP-class calcium channel antagonists (McCleskey, 1985). In agreement with these findings, either no change or an increase in caffeine-induced contractures has been found in various studies using DHP-class calcium channel ligands (Singh and Dryden, 1988; Viires et al., 1991). These studies further indicate that the depression of tetanic responses observed during our studies is not due to the changes in action potential properties or to the effect of these drugs on intracellular sites.

5.5 Depression of Tetanic Responses by Verapamil

Another organic calcium channel blocker used for further investigation of the functional roles of slow calcium channels was verapamil of the phenyalkylamine group. Depending on the concentration range of verapamil used, different effects on the mechanical and electrical properties of muscle fibres were observed. At relatively low (3×10^{-6} M) concentrations of verapamil, a significant depression of the area under the tetanic tension x time curve was observed. At these concentrations, verapamil neither blocked the intracellularly recorded repetitive action potentials nor blocked the twitch responses. With increasing concentrations of verapamil, however, repetitively occurring action potentials were blocked. The blockade of the

repetitive action potentials by higher concentrations of verapamil occurred in a use-dependent manner.

Similar results showing the depressant effects of verapamil on the tetanic contractions of mammalian muscle fibres have also been reported previously (Kotsias et al., 1986). In their study, using verapamil at 10^{-4} M and higher concentrations, Kotsias et al. (1986) reported that both the twitch and tetanic contractions were blocked. At such a high concentration, blockade of single action potentials has been reported to occur in numerous studies (Bondi, 1978; Marwaha and Treffers, 1980; and Frank, 1984).

Interestingly, although a significant ($\simeq$ 27%; n:5) decrease under the area of tetanic tension x time curve was observed in the presence of 3×10^{-6} M verapamil, no change or a slight decrease of the amplitudes of repetitive action potentials were recorded intracellularly (Figures 16, 21). These results indicate that repetitive action potentials remain intact, although, the late phase of tetanic contraction is not able to be maintained. These results give further support to suggestions that the entrance of extracellular calcium ions, presumably through VSCCs, can play a role during the late phase of tetanic contractions.

The results obtained in the presence of verapamil were more complicated than earlier findings with nitrendipine. Although we were careful to use only lower verapamil concentrations which did not block single twitches, still most of the concentrations we used did result in a use-dependent block of some of the action-potentials during the stimulus trains at 100 Hz for 2 sec (Frank and Oz, 1991). Even so, we were able to demonstrate a clear reduction of the tetanus area due to the block of VSCCs by verapamil under two experimental conditions. The first was a significant reduction of the area by 3×10^{-6} M verapamil (Figure 16) which was not a high enough concentration to produce a use-dependent action potential block at 100 Hz stimulation for 2 sec (Figure 21). The second was the reduction of the tetanus area produced by 5×10^{-6} M

verapamil (Figure 17) when the stimulus frequency was reduced to 50 Hz at which frequency this verapamil concentration did not produce any use-dependent action potential block (Figure 23).

In frog skeletal muscle, in addition to a block of the slow Ca^{2+} channels, verapamil at slightly higher concentrations also blocks Na^+ channels. Our results indicate that the repetitive firing of action potentials facilitates the blockade of Na^+ action potentials in frog skeletal muscle fibres. At the concentration range of 5×10^{-6} to 5×10^{-5} M, although no blockade of single action potentials was observed, repetitive production of action potentials at the stimulation frequency of 100 Hz, elicited the use-dependent blockade of Na^+ channels (see Figure 22). This use-dependent blockade of Na^+-channels was reversed with the use of lower stimulation frequencies such as 50 or 25 Hz. At the concentration of 5×10^{-6} M verapamil, using a 50 Hz stimulation frequency was enough to remove the blockade of Na^+ channels. On the other hand, with 10^{-5} M verapamil, a lower stimulation frequency of 25 Hz, which could produce only an unfused tetanus, was required to eliminate the blockade of Na^+ channels. At concentrations such as 5×10^{-6} to 10^{-5} M of verapamil, we sometimes also observed the blockade of Na^+ action potentials following a biphasic time course (see Figure 22). In about 0.5 to 1 sec, there was a complete blockade of action potentials and subsequent decrease of late after potentials. This was followed by a second activation of full size action potentials which lasted a few hundred milliseconds. This second activation of action potentials during 2 sec pulse stimulations, was also consistent with tension recordings in which a second rise in the tension curve was also observed (see Figure 16). These findings suggested that the blockade of the Na^+ channels by verapamil declined during the time between these two activation periods (see Figure 22). In addition to removal of the use-dependent blockade of Na^+ channels by the absence of action potentials, the gradually decreasing levels of late after potentials might also contribute to the removal of Na^+ channel inactivation and thus the production of a second burst of action potentials. The time

required for recovery from the use-dependent blockade of action potentials by verapamil was a few hundred milliseconds. This approximate recovery time is also quite similar to that seen with local anesthetics such as lidocaine that causes a use-dependent blockade of Na^+ channels (Hille, 1988). Similar effects of verapamil have also been observed on slow, soleus muscle fibres of rat (Kotsias and Muchnik, 1985). Thus, in addition to the "use-dependent" blockade of voltage sensitive slow Ca^{2+} channels by verapamil and D-600 (Lee and Tsien, 1983; Frank, 1986), this type of blockade and its reversal was only recently reported for Na^+ channels in frog twitch muscle fibres (Frank and Oz, 1991).

In conclusion, in the present study, different effects of verapamil have been observed on frog skeletal muscle. At relatively low concentrations, verapamil causes an inhibition of tetanic contractions by the blockade of voltage sensitive Ca^{2+} channels. At slightly higher concentrations, verapamil, in addition to its known use-dependent blockade of calcium channels, also produces a use-dependent blockade effect on Na^+ channels of frog's skeletal muscle.

5.6 $^{45}Ca^{2+}$ Flux Response Occurred in the Range of LAPs and its Pharmacological Characterization

The main findings of our studies on calcium flux responses is that transverse tubule vesicles isolated from rabbit skeletal muscle contain functional voltage-dependent calcium channels and that these calcium channels respond to potential changes that would be expected to occur under physiological conditions in mammalian muscle fibres. Previously, calcium channel activity in these membranes has been investigated after their reconstitution into planar bilayers (Affolter and Coronado, 1985; Coronado and Affolter, 1985) or into phospholipid vesicle preparations (Curtis and Catterall, 1986). New insights into the activity of skeletal muscle calcium channels have been obtained using these techniques, but only a few active channels may

be examined by this method. An advantage of using native vesicle preparations is that the channel population as a whole may be studied. Correlation of the properties of ligand binding to the membranes with their effects on the functional responses of the overall channel population is particularly crucial in the case of transverse tubule membranes in view of their high density of DHP binding sites (Fosset et al., 1983), the electrophysiological localization of voltage-dependent calcium channels in these membranes (Almers et al., 1981), and the frequent lack of correspondence between the two (see e.g. Schwartz et al., 1985).

In this study, transverse tubule membranes were prepared by a method previously described (Dunn, 1989). Properties of these vesicles related to the integrity of vesicles and their sidedness in this preparation, have been well established by previous studies (Hidalgo et al., 1986; Dunn, 1989). The preparation of transverse tubule membranes used here consist mainly of sealed vesicles that are oriented primarily inside-out (90% of vesicles).

Membrane potential changes of transverse tubule membranes in response to establishing potassium gradients across the membrane in the presence of valinomycin, have also been observed by monitoring the fluorescent changes of the voltage-sensitive dye, 3,3′-dipropyl-2,2′-thiodicarbocyanine (Dunn, 1989). In these experiments, it was shown that transverse tubule membranes are capable of developing and maintaining membrane potentials in response to inward and outward potassium gradients in the presence of valinomycin. These potentials are readily reversible and dissipate only slowly over a period of minutes to hours. Thus, transverse tubule membrane vesicles are suitable for study of voltage-dependent ion channels, since the membrane potential may be experimentally manipulated.

The importance of our experimental results comes from the fact that the potential range that was used to reactivate voltage sensitive calcium channels in transverse tubule membrane vesicles, has been confined to the physiological range of LAPs. In earlier electrophysiological

studies, it has been well established that these LAPs are due to the accumulation of K^+ ions passing through delayed potassium channels during repetitively developed action potentials (Freygang et al., 1964a,b; Gage and Eisenberg, 1969; Kirsch et al., 1977; Oz and Frank, 1991). Hence, flux responses which occurred at the range of LAPs in transverse tubule membrane vesicles may reflect the possible functional roles of calcium channels accumulated in transverse tubules of muscle fibres during repetitively developed action potentials, such as in tetanic contractions.

Following the experimental results showing the presence of calcium flux responses in transverse tubule vesicles at the range of LAPs, we further studied the pharmacological characterization of these flux responses.

Suppression of calcium currents by various inorganic calcium channel blockers, in millimolar concentrations, has been shown in numerous studies in mammalian and vertebrate skeletal muscle fibres (Donaldson and Beam, 1983; Palade and Almers, 1985; Cota and Stefani, 1986). In line with previous observations, all of the inorganic calcium channel blockers, Co^{2+}, Ni^{2+} and La^{3+}, used in similar concentration range, also inhibited calcium flux responses elicited by partial depolarization in transverse tubule vesicles used in this study. Although the inhibition of calcium fluxes by Co^{2+} (2 mM) and Ni^{2+} (2 mM) was partial, La^{3+} (1 mM) completely blocked the calcium fluxes in transverse tubule vesicles. La^{3+} has shown to be the most potent blocker of calcium channels in different muscle preparations, including barnacle skeletal muscle (Hagiwara, 1973, 1975), cardiac muscle (Lansman et al., 1986) and smooth muscle (Anderson, et al., 1971). An interesting property of blockade of calcium fluxes by Co^{2+} and Ni^{2+} was that simultaneous application of these inorganic calcium channel blockers did potentiate each other's effect, indicating more than simple summation of two different routes of calcium flux. T-type calcium channels in different preparations have been found to be preferentially sensitive to block

by Ni^{2+} but resistant to block by Cd^{2+} (Nowycky et al., 1985; Narahashi et al., 1988; Hagiwara et al., 1988). Unfortunately, although the existence of T-type like 'fast' calcium channels has been reported in vertebrate skeletal muscle fibres (Cota and Stefani, 1986), the pharmacological properties of this current have not been studied in detail (Beaty et al., 1987). Our results showing the potentiating effect of simultaneous application of Ni^{2+} and Co^{2+} are consistent with the concept of two different populations of calcium channels in skeletal muscle fibres each with unique spectrum of pharmacological susceptibilities (Beaty et al, 1987).

Organic calcium channel antagonists have been shown to cause the blockade of calcium currents in skeletal muscle fibres in numerous studies (Walsh et al., 1986, 1988; Cognard et al., 1986). The concentration of organic calcium channel antagonists required to block calcium currents is usually 1 to 2 order higher than the concentration required in cardiac or smooth muscle (Triggle and Janis, 1987). In agreement with these observations, we have also found that in a similar concentration range, verapamil and nifedipine blocked the flux response induced by partial depolarization, albeit partially. On the other hand, nitrendipine had no effect on this calcium flux. In rabbit skeletal muscle fibres, Walsh et al. (1986) also found no effect of nitrendipine on calcium current at this concentration range. Based on these results, it appears that nitrendipine does not show any antagonist effect on calcium channels of mammalian muscle. On the other hand, the effect of nitrendipine on frog skeletal muscle fibre has reported to be antagonistic on calcium currents and on high K^+ induced contractures (Schwartz et al., 1985; Frank, 1990). Consistent with these previous results, during our studies, nitrendipine had a depressant effect on tension development during tetanic contractions in frog's skeletal muscle fibres. But in $^{45}Ca^{2+}$ flux studies that are performed on rabbit muscle membranes, nitrendipine had no antagonistic effect.

We have also conducted some experiments to observe the effect of (±) Bay K8644 on

these flux responses. It was found that, in the presence of 1 μM ($\pm$) Bay K8644, there was a significant increase of $^{45}Ca^{2+}$ efflux from T-tubule membrane vesicles. The increasing effect of Bay K8644 on $^{45}Ca^{2+}$ flux response was also observed in control conditions, without repolarizing and partial depolarizing buffer applications. Since in control conditions the concentration of potassium ions is equal in the intra- and extravesicular compartments, the potassium equilibrium potential that determines the potential difference across the vesicular membranes is zero; i.e. there is no potential difference across the vesicle membranes, and VSCCs in vesicle membranes are in an inactivated state. In a number of studies it has been shown that the affinity of DHP-class calcium channel ligands is voltage dependent and increases up to 3 orders in magnitude in depolarized membranes (Bean, 1984; Sanguinetti and Kass, 1984; Godfraind et al., 1986). Although it is not possible to observe the antagonistic effect of channel ligands, on $^{45}Ca^{2+}$ flux since there is no Ca^{2+} flux through inactivated channels by which an antagonistic effect would be detected, agonistic effects can easily be detected. Thus in controls, i.e. only depolarized conditions, as well as in repolarized vesicles, the calcium channel agonist, Bay K8644 significantly increased calcium flux responses.

The stereospecificity of DHP effects has proved to be a useful tool in the study of calcium channel properties (Franckowiak et al., 1985; Hof et al., 1985) and, in most studies, both channel activators and inhibitors have been shown to compete for the same binding sites (Williams et al., 1985; Kokubun et al., 1986). In depolarized, intact cardiac cells, for example, both a channel activator, (+)-SDZ 202-791, and the blocker, S-isradipine, appear to bind in a competitive manner (Kokubun et al., 1986). In the present study, the isomers (-)-SDZ 202-791 and (+)-SDZ 202-791 were found to have opposite effects on calcium flux responses. These results provide further support for the notion that the T-tubular calcium flux responses occurring in the range of LAPs are mediated, in part at least, by VSCCs.

5.7 Proposed Mechanism For the Functional Role of VSCCs During Tetanic Contractions

In order to probe the functional role of VSCCs during maintained contractions like tetanus, we have used some of the available pharmacological tools. In summary, using 10 min exposure time with EDTA containing Ca^{2+}-free Ringer's solution, we were able to observe large decreases under the area of tetanic tension x time curve. At the concentration range of EDTA used in Ringer's solution (less than 2.5×10^{-4} M), there was no decrease in the maximum amplitudes of twitches. Similarly nitrendipine at the concentration range of 10^{-6} to 10^{-4} M and verapamil at the concentration of 3×10^{-6} M greatly depressed the area under tetanic tension x time curve, without affecting repetitive or single action potentials or twitch amplitudes. Indeed, the maximum amplitudes of twitch tension were significantly increased with both nitrendipine and verapamil applications.

These results indicate strongly that the depression of the late phase of tetanic contractions are due to inhibition of slowly developing L-type calcium current in frog skeletal muscle fibres.

Electrophysiological studies on the activation kinetics of L-type VSCCs has crucial importance on the interpretation of our results. In frog skeletal muscle fibres, detailed kinetic analysis of calcium currents using voltage-clamp conditions have been performed in various studies (Almers and Palade, 1981; Sanchez and Stefani, 1983). It was reported by Sanchez and Stefani that for a depolarization from -90 mV holding potential to 0 mV, time to peak Ca^{2+} current was 100 - 200 msec in frog twitch muscle fibres. Similar values have also been obtained in different studies (Stanfield, 1977; Sanchez and Stefani, 1983). An obvious conclusion from these studies is that during a single action potential that lasts only 1 - 2 msec, only a few percent of all available L-type VSCCs and a certain amount of fastCa^{2+} channels (activation time constant $\simeq$ 5 msec; (Cota and Stefani, 1986) can be activated, and any activated calcium channels close quickly upon repolarization (time constant of $\simeq$ 150 msec) as opposed to a few msec in neurons

(Almers et al., 1985). Hence, judging from the previously published experiments on kinetic properties of VSCCs, it seems that these channels have no obvious role during a single action potential. However, all the voltage-clamp studies so far have been carried out under unphysiological conditions (see also Almers et al., 1985), and the observed gating kinetics may be similarly unphysiological. Ca^{2+} channels may gate more rapidly at lower external [Ca^{2+}] (Almers et al., 1981), or with higher temperatures in mammals. For example, increasing the temperature from 22 °C to 32 °C results in a doubling of the amplitude of slow calcium currents and a 4-fold decrease in the time to peak conductance measurements in rabbit skeletal muscle fibres (Walsh et al., 1986). Finally, Donaldson and Beam (1983) have suggested that in rat skeletal muscle fibres, slow calcium channels may gate at sufficient speed at 37 °C to enable a significant number of them to respond to a single action potential. Furthermore, the lack of some of the soluble messengers in the cytosol would also affect the activation kinetics and the number of channels available for activation by depolarization pulses (Areola et al., 1987; Kokate et al., 1992; Nunoki et al., 1989). Also, the observed $^{45}Ca^{2+}$ influx per action potential ($\simeq$ 1 pmol/cm^2; Curtis, 1966) in intact muscle fibres, is an order of magnitude larger than influx through calcium channels predicted on the basis of voltage-clamp studies (Almers and Palade, 1981; Sanchez and Stefani, 1983; see also Almers et al., 1985). Although the entrance of a small amount of extracellular Ca^{2+} through fast Ca^{2+} channels (Beaty et al., 1987) has also been suggested, it has been well established in numerous studies that the entrance of extracellular Ca^{2+} through VSCCs is not required for the twitch responses (Armstrong et al., 1972; Frank, 1982; Brum et al., 1988).

On the other hand, in maintained depolarizations such as voltage pulses under voltage-clamp conditions or by high K^+-induced depolarizations, it was shown that these slow calcium channels can be activated and cause the entrance of extracellular Ca^{2+} into the muscle fibres

(Sanchez and Stefani, 1983; Frank, 1984). Similar maintained depolarizations of 20 - 25 mV in amplitude have also been reported during repetitive stimulations of muscle fibres (Freygang et al., 1964; Kirsch et al., 1977). During repetitive action potential trains, the accumulation of potassium ions flowing through outwardly rectifying, delayed K^+ channels, has been reported in numerous studies (Freygang et al., 1964a,b; Hellam et al., 1965). Since the glycerol-removal treatments of skeletal muscle fibres abolishes the development of early and late after-potentials (see Introduction, section 1.3.2), it was suggested that these late after-potentials are originated from T-tubule systems (Gage and Eisenberg, 1969a,b). It was also shown that in solutions in which the tonicity was increased twice with sucrose, the time of decrement (measured as the fall from 6 mV to 3 mV) is prolonged about 4-fold with a negligible change in amplitude (Freygang et al., 1964b). The LAP is considerably reduced if the majority of intracellular K^+ ions is exchanged for Rb ions, to which, T-tubules are impermeable (Hellam et al., 1965). These results support the suggestion that LAPs are located in T-tubules and originate from the accumulation of potassium ions during repetitive stimulation of muscle fibres. These LAPs at an amplitude of 20-25 mV would cause the opening of VSCCs.

At the range of LAPs, it has been reported in numerous studies that there is a small but continued calcium influx that can cause an increase of $[Ca^{2+}]_i$ (Bianchi and Shanes, 1959; Weiss and Bianchi, 1965; Blatter and Blinks, 1991). As early as 1931, Fenn showed that the respiration of frog twitch muscles increases when $[K^+]_o$ increased to 20-30 mM. In these conditions, the total heat production by the muscle also rises, as was described by Solandt (1936). The increase in metabolism produced by elevated $[K^+]_o$ is often called the Solandt effect (Solandt, 1936). Later, Frank and Vos (1972a,b) found that the resistance of muscle fibres to stretching, which is related to the number of cross-linkings between actin and myosin filaments, increases with elevated extracellular potassium concentrations to the mechanical subthreshold levels. The

destruction of the T-tubule system by the glycerol-removal technique eliminates potassium-stimulated respiration (Van Der Kloot, 1969). Furthermore, the increase in potassium-stimulated respiration and $[Ca^{2+}]_i$ was found to be dependent ultimately upon a supply of extracellular Ca^{2+} (Novotny and Vyskocil, 1966; Van Der Kloot, 1969). In addition, Chirandini and Bentley (1973) have also reported that verapamil greatly decreased this potassium-stimulated oxygen consumption.

Interestingly, in a recent study, it was reported that sustained-subthreshold depolarizations causes a slowly developing sustained increase of intracellular calcium levels and contractions in mammalian ventricular myocytes (Talo et al., 1990). Nitrendipine, verapamil, Cd^{2+} and Ni^{2+} abolished these steady contractions.

In addition to these previous observations, we have also taken a biochemical approach to investigate further this calcium transport process which occurs at the range of LAPs. Due to the difficulties of the voltage-clamping of T-tubules deep in the muscle fibres, we have used the T-tubule membrane preparations. T-tubule membrane preparations have been shown to have DHP-binding proteins that are presumably L-type VSCCs (Fosset et al., 1983; Dunn, 1989). It was also reported that these L-type VSCCs transport $^{45}Ca^{2+}$ in a voltage dependent manner (Dunn, 1989). Furthermore, the pharmacological and functional characterization of these VSCCs has also been well established (Dunn, 1989; Bhat et al., 1992). At the range of LAPs, we have observed large $^{45}Ca^{2+}$ fluxes through T-tubule membrane vesicles. In addition, inorganic calcium channel blockers such as Co^{2+} (2 mM), Ni^{2+} (2 mM) and La^{3+} (1 mM) significantly blocked these flux responses. These results strongly suggested that during tetanic stimulations, at the range of LAPs there is an entrance of extracellular Ca^{2+} through L-type VSCCs into the muscle fibres.

As important as the LAPs, are the changes in the kinetic and gating properties of L-type

VSCCs during repetitive action potentials. In a number of studies, it was found that repetitive stimulations increase the activation rate of calcium currents in frog skeletal muscle fibres (Garcia et al., 1990a), in mammalian ventricular myocytes (Noble and Shimoni, 1981; Schouten and Morad, 1989) and in adrenal chromaffin cells (Fenwick et al., 1982). Interestingly, Garcia et al. (1990a) found also that this increased activation rate of calcium currents was pronounced when the potential was held at -50 mV between conditioning and test pulses. Since this potential level is also in the range of LAPs, it is also possible that in intact muscle fibres, the presence of LAPs would further facilitate the increased activation rate of calcium channels. Furthermore, the increased duration of action potentials in T-tubules has also been reported in some studies using optical recording techniques (Heiny and Vergara, 1982; Vergara and Delay, 1986). This prolonged duration of action potentials would also cause the increased number of channels being activated. Very recently, in Dr. Vergara's laboratory, using membrane impermeable voltage sensitive dyes, it was found that these prolonged T-tubular action potentials significantly summate when frog skeletal muscle fibres stimulated at 60 Hz frequency (Dr. Vergara's unpublished data). In summary, the additive effects of the summation of prolonged T-tubule action potentials and LAPs during repetitive stimulations would supply the amount of maintained depolarization levels that is necessary to open these VSCCs located in T-tubules. Interestingly, in a recent study digitally constructed wave forms that stimulate natural action potentials were used as voltage-clamp commands in dorsal root ganglion neurons (McCobb and Beam, 1991). It was found that during the recordings of whole cell calcium currents elicited by these "action potentials", the entry of extracellular calcium ions through high-voltage activated channels was highly responsive to the duration of action potential, increasing more significantly as action potential duration increased (McCobb and Beam, 1991). It is also known that the potential change, although it is essential, is not the only parameter affecting the activity of VSCCs in skeletal muscle fibres. The

modulation of the activity of calcium channels by cAMP, protein kinase-A, GTP-γ-S, IP_3 and other intracellular components of signal transmission has been reported (Arreola et al., 1987; Yatani et al., 1988; Vilven and Coronado, 1988; Garcia et al., 1990b; Kokate et al., 1992; see also Introduction section 1.6.5). Combined effects of these intracellular components may also increase the activity of these channels significantly during repetitive activities in in situ conditions.

These experimental observations discussed so far shed some light on the possible involvement of L-type VSCCs during tetanic contractions, and give further support to our hypothesis that during tetanic contractions, extracellular calcium ions passing through VSCCs that are opened by LAP and repetitive action potentials, are required for the maintenance of the late phase of tetanic contractions.

5.8 Future Recommendations to Pursue This Project

Our experimental results, based on pharmacological and biochemical studies, strongly suggest that the L-type VSCCs are required to maintain tetanic contractions. Although these results are very suggestive, some further studies on the development of repetitive action potentials in T-tubules and also on the measurement of calcium currents would be necessary to find more definite answers to the question of what the functional roles of VSCCs are in T-tubules of skeletal muscle fibres. Firstly, to our knowledge, there is no experimental study reporting the time courses of potential changes during repetitive stimulations in T-tubules. Since intracellularly recorded repetitive or single action potentials reflect the potential changes occurring mainly on the sarcolemmal surface of the muscle fibres, potential changes on T-tubules cannot be recorded by any of the intracellular potential recording techniques. On the other hand, using nonpermeant potential sensitive dyes, it has been possible to study the potential changes across the T-tubule

membranes (Nakajima and Gilai, 1980a,b; Heiny and Vergara, 1982). Thus the use of these optical signals from T-tubule membranes would enable us to observe the potential changes in T-tubules during repetitive stimulations.

Having these experimental results obtained by using membrane impermeable voltage sensitive dyes on the time courses of the potential changes in T-tubules during repetitive stimulations, would also help us to design future electrophysiological experiments more accurately. Primary cultures of myotubes prepared from skeletal muscle fibres of newborn mammals (Beam and Knudson, 1988) have extensively been used for electrophysiological studies. Since these cultured myotubes have no T-tubule system, they appear to be very suitable for voltage-clamp conditions that require the isopotential distribution throughout the cell surface. Whole-cell configuration of the patch clamp technique has been applied to these cells by various groups (Beam and Knudson, 1988; Cognard et al., 1990). Calcium currents and their pharmacological modulations have been subject to many studies using patch clamp techniques in these cultured cells (Rivet et al., 1989; Adams and Beam, 1991). But in these studies, only long lasting (0.2 - 1 sec) square pulse voltage-clamp protocols have been applied to observe calcium currents. On the other hand, applying the digitally constructed wave forms that can simulate the time course of repetitive action potentials recorded by membrane impermeable voltage sensitive dyes (more accurately, actual potential changes across the T-tubule membranes) would greatly help the recording of calcium currents under relatively physiological depolarizing voltage-clamp pulse-protocol conditions. Pharmacological characterization of such a calcium current, if there is any, would also be useful for the further understanding of VSCCs in skeletal muscle fibres.

In addition to their blocking effects of VSCCs, organic calcium channel blockers have also been shown to have various intracellular effects (Zernig and Glossmann, 1988). Among them, intracellular Ca^{2+} release sites such as terminal cisternae or heavy SR membrane

preparations have been used to investigate the effects of organic calcium channel blockers on intracellular calcium release sites (Wang, et al., 1984; Su, 1988). So far, in various studies, different results have been reported (see Discussion section, 5.3). It would be important to investigate further the effects of organic calcium channel blockers on $^{45}Ca^{2+}$ effluxes in heavy SR membrane vesicles loaded with $^{45}Ca^{2+}$.

6. SUMMARY AND CONCLUSIONS

1. The effects of Ca^{2+}-free Ringer's solution were tested on the tetanic contractions elicited by 100 Hz stimulation frequency for 2 sec, and twitch responses. In a 10 min exposure to Ca^{2+}-free Ringer's solution containing EDTA at concentrations that are less than 2.5 x 10^{-4} M, the area under the tetanic contraction x time curve was greatly depressed. But twitch responses were either potentiated or remained unchanged.

2. Organic calcium channel blockers, nitrendipine and verapamil were also tested for their effects on twitches and tetanic contractions. It was found that nitrendipine (10^{-6} - 10^{-4} M) and verapamil (3 x 10^{-6} M) reduced the tetanic area without reducing twitch responses. Except at very high concentrations of verapamil (10^{-4} M), both nitrendipine and verapamil at all concentrations significantly increased the maximum amplitudes of twitch responses.

3. During intracellular recordings of repetitive action potentials, stimulated at the same frequency (100 Hz, 2 sec) and for the same duration (2 sec) as was used in mechanical recordings, it was found that nitrendipine does not block these repetitively occurring action potentials.

4. The effect of verapamil on intracellularly recorded repetitive action potentials was of a more complex nature. At a low concentration of verapamil (3 x 10^{-6} M), repetitively occurring action potentials remained intact. With increasing concentrations of verapamil (5 x 10^{-6} to 10^{-5} M), there was a concentration and use-dependent blockade of repetitive action potentials.

5. At the end of the repetitive action potential trains, a relatively large LAP at an amplitude of 20 - 25 mV was measured intracellularly. The amplitudes of these LAPs were not affected by nitrendipine or verapamil at the concentrations at which these drugs caused large decreases in the tetanic tension x time area.

6. At the range of LAPs, significant $^{45}Ca^{2+}$ effluxes in $^{45}Ca^{2+}$ loaded T-tubule membrane vesicles were observed.

7. All inorganic calcium channel blockers, Co^{2+}, Ni^{2+} and La^{3+} significantly blocked these flux responses that are elicited at the range of LAPs. Among the organic calcium channel antagonists, nitrendipine (10^{-5} M) had no effect on these flux responses. On the other hand, other DHP-class calcium channel antagonists including nifedipine and (-)-SDZ 202-791, and verapamil, from the phenylalkylamine group, were able to block these calcium flux responses to varying degrees. Stereospecificity of the effects of DHPs were also investigated using stereospecific enantiomers of (±)-SDZ 202-791. It was found that whereas the (-)-SDZ 202-791 enantiomer blocked these calcium flux responses, the (+) enantiomer of SDZ 202-791 had an agonistic effect, further increasing the efflux responses.

8. The effects of the DHP class agonists such as (±) Bay K8644 were also investigated in calcium flux responses. It was found that (±) Bay K8644 had significant stimulatory effects on calcium flux responses. This stimulatory effect of (±) Bay K8644 also occurred during the inactivated state of calcium channels in T-tubule membrane vesicles.

9. It was concluded that the voltage-sensitive, slow Ca^{2+} channels are opened by the depolarization produced by the accumulation of K^+ ions in the T-tubules and by repetitive action potentials during tetanic contraction. It was also concluded that since isolated twitches are rarely, if ever, seen in functioning skeletal muscle *in situ*, and bursts of action potentials lasting more than 0.5 sec are not rare, it was suggested that these voltage-sensitive, slow Ca^{2+} channels play an important role in the normal physiological functioning of skeletal muscle fibres.

7. REFERENCES

The ancient covenant is in pieces; man knows at last he is alone in the universe's unfeeling immensity, out of which he emerged only by chance. His destiny is nowhere spelled out, nor is his duty. The kingdom above or the darkness below: It is for him to choose.

- Jacques Monod

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